

Research Article

Native and non-native unionids respond differently to the presence of fouling dreissenid mussels

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Abstract

Unionid mussels are globally threatened by several human disturbances, including the introduction of non-native species. Among these, biofouling zebra and quagga mussels of Ponto-Caspian origin are considered to be especially detrimental to unionid locomotion, filtration and physical condition. The aim of our study was to determine and compare the impact of dreissenid fouling and/or presence on locomotion and burrowing of the native Unio tumidus and invasive Sinanodonta woodiana, a novel invader expanding its range in Europe in recent decades. We tested unionids collected from Lake Balaton (central Europe) that were fouled by dreissenids (zebra and quagga mussels mixed), cleaned of fouling or non-fouled (collected without any signs of dreissenid fouling). Moreover, unionids were tested in the presence or absence of other fouled individuals and dreissenids isolated in mesh bags to determine the influence of direct fouling and presence of dreissenids in the environment on unionid behaviour. Movement initiation time, locomotion distance and burrowing level were retrieved from videos recorded for 24 hours. Direct fouling affected only the behaviour of U. tumidus, limiting their burrowing and delaying movements. After removal of fouling, movement timing returned back to normal, but mussels still burrowed less than the control non-fouled individuals, indicating persisting effects of fouling on physical condition. Moreover, U. tumidus reduced their locomotion in the presence of fouled unionids. Sinanodonta woodiana responded to the presence of dreissenids (especially quagga mussels) with increased burrowing. These different responses of the two unionid species to Dreissena spp. indicate that biofoulers may influence biotic interactions between the unionids by promoting the invasive species (less susceptible to negative effects of fouling). Moreover, S. woodiana may indirectly affect U. tumidus through apparent competition, constituting an environmental reservoir of biofoulers exerting a stronger impact on the native species.

Key words: Biofouling, biological invasions, Bivalvia, burrowing, ecosystem engineers, interspecific interactions, locomotion, *Sinanodonta woodiana, Unio tumidus*, Unionidae

Introduction

Bivalves are ecosystem engineers altering ecosystem structure and function by increasing water clarity and modifying the bottom quality (Strayer et al. 1999; Karatayev et al. 2002; Sousa et al. 2009). Their reported effects consist in declines in plankton abundance (Kissman et al. 2010; Karatayev et al. 2023), decreases in



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young fish survival (Irwin et al. 2009), changes in fish feeding patterns (Mayer et al. 2001; Balogh et al. 2022), increases in benthic invertebrate abundance (Novais et al. 2015; Ilarri et al. 2018), and development of submerged aquatic vegetation (Chu et al. 2004; Zhu et al. 2006), resulting in a more benthic-oriented food web structure (Mills et al. 2003; Miehls et al. 2009).

Bivalve species richness and abundance decrease all over the world due to ongoing climate change, increasing water pollution and habitat destruction (Strayer and Malcom 2007). This particularly applies to freshwater mussels of the family Unionidae, with dozens of already extinct species and hundreds more heavily endangered (Aldridge et al. 2023; Sousa et al. 2023). Due to their important environmental functions and ecosystem services they provide, further unionid extinction may lead to irreversible changes and effects on the entire aquatic community (Vaughn 2018; Zieritz et al. 2022).

One of the greatest threats to native unionid mussels is the spread of invasive mussel species (Gutiérrez et al. 2014; Douda et al. 2024), including biofouling dreissenids: the zebra mussel *Dreissena polymorpha* (Pallas, 1771) and the quagga mussel *Dreissena rostriformis bugensis* Andrusov, 1897. The dreissenids, originating from the Ponto-Caspian region (catchments of the Black, Azov and Caspian Seas and lowland parts of the inflowing rivers) have successfully invaded freshwater ecosystems in Europe and North America (Matthews et al. 2014; Collas et al. 2018). *Dreissena polymorpha* has spread in Western Europe since the beginning of the 19th century (Harzhauser and Mandic 2010), whereas *D. r. bugensis* started to spread in the 20th century (Therriault et al. 2005) and its invasion is ongoing on both continents. This has led to the co-occurrence of both species in more and more invaded locations (Zhulidov et al. 2004; Grutters et al. 2012).

Dreissena spp. rapidly develop large populations and settle on unionids in high numbers, especially when other hard substrata are scarce, and the mass of fouling dreissenids can exceed the biomass of their host (Burlakova et al. 2000). This impairs physiological condition of unionids, as *Dreissena* spp. can block their siphons preventing them from filtering and therefore breathing and feeding (Ricciardi et al. 1996; Sousa et al. 2011; Bódis et al. 2014). Dreissenids can also inhibit unionid locomotion (Van Appledorn and Bach 2007) and/or burrowing, making them vulnerable to unfavourable environmental conditions or predation (Saloom and Duncan 2005). In addition, they may compete for food with the unionids, causing severe declines in phytoplankton (Higgins and Zanden 2010). As a result, the spread of *Dreissena* species may further endanger or lead to extinction of already threatened unionid species (Ricciardi and Whoriskey 2004).

An exception in the generally endangered Unionidae family is the invasive Chinese pond mussel *Sinanodonta woodiana* (Lea, 1834), native to Eastern Asia (Lopes-Lima et al. 2020), but in recent decades (since 1959) spreading in Europe (Douda et al. 2024). This is a fast-growing and fast-reproducing (Douda et al. 2021) species of high ecological plasticity, tolerant to wider ranges of environmental conditions in comparison to native European Unionidae (Kraszewski and Zdanowski 2007; Poznańska-Kakareko et al. 2021; Zieritz et al. 2021; Dobler et al. 2022). It can co-occur with native mussels and outcompete them for food, fish hosts for their larvae, and living space (Douda and Čadková 2018).

Both native and invasive Unionidae are commonly fouled by dreissenids (Bódis et al. 2014; Balogh et al. 2024). Dreissenids can interfere with the invasion of *S. wood-iana*, with the final outcome depending on the relative susceptibility of *S. woodiana*

and native unionids to fouling and its effects (Bódis et al. 2014). High vulnerability of the invasive S. woodiana to fouling would negatively affect its establishment in areas occupied by dreissenids, whereas its resistance would facilitate competitive exclusion of other unionid species (Sousa et al. 2011). Therefore, the aim of our study was to determine and compare the effects of fouling and/or presence of the invasive mussels D. r. bugensis and D. polymorpha (responses to own fouling, to the presence of fouled conspecifics and to the presence of dreissenids on other substratum in the environment) on the locomotion and burrowing of two unionid mussels: the native Unio tumidus Philipsson, 1788 and the invasive S. woodiana. These species often co-occur in areas invaded by S. woodiana in Europe (e.g. in Lake Balaton; Benkő-Kiss et al. 2013) and can interact with each other, which can be additionally modulated by biofouling. For our study, the mussels were collected from a single location in Lake Balaton (Hungary) to make sure that potential differences in local living conditions of particular species would not affect the results. We hypothesized that: (1) fouling reduces unionid locomotion and burrowing; (2) this effect can be purely mechanical (only when the fouling is directly present), or (3) can persist after fouling removal (due to worsened physiological condition of the host). Additionally, we hypothesized that (4) unionids can detect and respond by behaviour to chemical cues released by other unionids fouled by dreissenids or directly by Dreissena spp. individuals.

Materials and methods

Mussel collection in the field

We collected all mussels manually (randomly by hand) or using a Surber net from Lake Balaton in Keszthely, Hungary (46°46'11"N, 17°14'53"E) in summer 2022. Lake Balaton, the largest lake in Central Europe, had been an isolated water body until the opening of the Sió Canal, which created conditions for the spread of invasive species from the River Danube (Benkő-Kiss et al. 2013). This eutrophic, shallow, and mainly muddy lake provides an opportunity to collect all the mussel species at one site: S. woodiana (temperate lineage from Eastern Asia) (Douda et al. 2024) has been present in the lake since 2006 (Benkő-Kiss et al. 2013), zebra mussels since 1932 (Sebestyén 1938) and quagga mussels since 2008 (Balogh et al. 2018). We collected S. woodiana and U. tumidus from sandy/muddy bottom at a water depth of 0.5-1 m. Some individuals were fouled with a mixture of both Dreissena species, whereas others were clean (without any marks of fouling). Dreissena polymorpha to D. r. bugensis ratio on unionid mussels at the collection site is ca. 3:2 (Balogh et al. 2024). We transported the mussels in buckets (containing sand and water from the collection place) to the laboratory in the Balaton Limnological Research Institute (Tihany, Hungary), where, after two weeks of acclimation, we conducted the experiments. We did not observe any mortality during this time. After completing all the tests, the mussels were weighed and measured (Table 1). We determined the mass of the Dreissena spp. fouling on each unionid considering it as a whole, without separating the dreissenid species. We assumed that the total fouling mass has the greatest effect on the fouled animal, irrespective of the fouling species, individual size and numbers. The fouling to host mass ratios were similar for both unionid species (Table 1) and indicated moderate level of fouling (20% of the host mass) compared to other environments, where the total fouling mass may even exceed that of their host (Burlakova et al. 2000; Dzierżyńska-Białończyk et al. 2018a).

	S. woodiana			U. tumidus			
	mean	SD	range	mean	SD	range	
Wet mass (g)	149.4	46.4	52.4-285.0	41.4	8.3	18.8–64.6	
Length (mm)	107.1	10.9	78–129	68.2	5.5	56-83	
Dreissena spp. mass (g)	27.0	11.5	10.5-50.0	7.5	4.6	4.0-20.4	
Dreissena / unionid mass ratio	0.20	0.08	0.06-0.33	0.17	0.08	0.09-0.32	

Table 1. Weight and length of native and invasive mussels used in experiments. Underlying data areavailable in Suppl. material 1.

SD – standard deviation.

Laboratory conditions

The laboratory with stock and experimental tanks was equipped with air conditioning to stabilise water temperature. Unionids, each species as well as fouled and non-fouled individuals separately, were stocked in aerated and filtered flow-through 250-L tanks supplied with fresh water from the lake (flow rate: 30 L/h). Water parameters in the stock and experimental tanks were measured with a multimeter Multi340i (WTW, Weilheim, Germany) (Table 2). The mussels were fed with cultured *Scenedesmus* sp. suspension at a concentration of 5 mg L⁻¹ (Douda and Čadková 2018) three times a week, also between the replicates of the experiments.

	Oxygen concentration [mg/mL]	Oxygen saturation [%]	Temperature [°C]	рН	Conductivity [µS/cm]
Stock tank	s				
Mean	8.90	98.33	19.26	8.60	855.67
SD	0.27	1.86	0.32	0.03	10.12
Range	8.73–9.21	96.6–100.3	18.94–19.57	8.57-8.63	844-862
Experimen	ital tanks		· · · · · · · · · · · · · · · · · · ·		
Mean	6.34	69.28	18.65	8.14	853.50
SD	0.49	5.22	0.30	0.21	21.33
Range	5.04-6.75	55.7-74.3	18.28-18.97	7.77-8.39	811-871

Table 2. Water parameters in the stock and experimental tanks (lake water).

Experimental setup

Direct impact of *Dreissena* spp. fouling on unionid behaviour (Experiment 1) was tested in $30 \times 30 \times 30$ cm tanks, containing 10 cm of sand (sifted and rinsed) covered by 10 cm layer of lake water. To each tank, we introduced 4 mussels: two individuals of *S. woodiana* and two individuals of *U. turnidus* (to mimic their co-existence in the field) (Fig. 1). The mussel behaviour was recorded for 24 h with CCTV video-cameras (Samsung SNB-6004, South Korea) equipped with infrared illumination (LIR-CS32, IRLAB, Taiwan) enabling night-time observations. We used several mussel variants depending on their fouling status and processing in the laboratory. Their full descriptions and explanations of use are presented in Table 3.



Figure 1. Mussel arrangement in the experimental tanks of Experiment 1 on the effect of *Dreissena* spp. fouling on the behaviour of *S. woodiana* and *U. tumidus*. See Table 3 for detailed explanations of variants (NF, F, F/C1, F/C2, CwF).

Table 3. Full list of variants and their comparisons in Experiment 1 with explanations of their purposes. See Fig. 1 for variant a	rrange
ments in the experimental tanks. In variants F, F/C1 and F/C2, we used the same individuals tested repeatedly.	

v	Variants		Description	Purpose		
NF (nor	NF (non-fouled)		Control; non-fouled unionids collected in the field without any trace of fouling on their shells	Control for the fouling variants (fouled F and cleaned F/C1)		
F (foule	F (fouled)		Fouled unionids tested with their fouling	To determine the direct impact of dreissenids fouling on mussel behaviour (compared to NF)		
F/C1 (cleaned, time 1)		time 1)	F unionids cleaned of fouling and tested for the second time two days after cleaning (with no dreissenids in the tank)	To determine whether the behaviour of mussels would change after the removal of fouling (compared to F) and whether it would return back to normal (compared to NF)		
F/C2 (cleaned, time 2)		time 2)	F/C1 unionids tested once again on the next day (for the third time in general, second time without fouling) To check whether the repeated testing of mussels and passin an impact on their behaviour (compared to F/C1)			
CwF (cl fouled)	CwF (cleaned with fouled)		Unionids collected in the field as fouled, cleaned of fouling and tested two days after cleaning in the presence of other, fouled unionids (from variant F)	To determine whether the presence of other fouled unionids has an impact on their behaviour (compared to F/C1, exposed in the total absence of dreissenids)		
Cor betwee	npariso n the va	ons ariants		Purpose		
NF	vs	F	To test the direct effect of fouling on unionid behavio	ur of both chemical cues and physical presence of biofoulers		
NF	vs	F/C1	To test the effect of past fouling on unionid behaviour	(potentially deteriorated condition of mussels recently cleaned of fouling)		
F	vs	F/C1	To test the possibility of potential recovery from the p	ast fouling with passing time		
F/C1	vs	F/C2	To test the potential effects of passing time on the resp	ponses of unionids exposed to the experimental conditions		
CwF	vs	F/C1	To test the unionid responses to the presence of fouled biofoulers)	d individuals in the environment (chemical cues from fouled unionids and		

First, we tested non-fouled mussels of variant NF (one *S. woodiana* and one *U. tumidus* individual per tank) accompanied by two additional non-fouled individuals (one per species) to have the total number of four individuals per tank. Then, we tested two fouled mussels (F) accompanied by two mussels cleaned two days before the exposure (CwF). After this exposure, fouled individuals (F) were cleaned and kept in tanks for two days. Then, they were exposed again (as F/C1) in the presence of two additional non-fouled mussels. On the next day, F/C1 mussels were exposed once again (as F/C2) in the presence of two additional non-fouled mussels. Thus, each experimental tank always contained four mussels, two of each species, in different fouling conditions (Fig. 1).

Effects of the presence of *Dreissena* spp. (Experiment 2) on unionid behaviour were tested in tanks prepared similarly as for Experiment 1. We introduced two cleaned (two days before the experiment) unionids (one *S. woodiana* and one *U. tumidus*) into each tank (one individual in the centre of each half of the tank bottom). We used cleaned mussels to be sure they had some past experiences with *Dreissena* spp. fouling. In one randomly selected corner of the tank, we placed a mesh bag with 50 individuals of *D. r. bugensis* (mean wet mass: 13.5–15 g) or *D. polymorpha* (12–12.5 g). Here, the two species of *Dreissena* spp. were tested separately, as, in contrast to the direct impact of the mass of attached individuals, we expected that unionids can respond differently to the remote presence of a particular fouler species. In parallel, we carried out control trials in tanks without mesh bags with dreissenids. Our Experiment 1 showed that the fouling *Dreissena* spp. mass of the magnitude used in Experiment 2 was capable of triggering behavioural responses of Unionidae (see Results).

All configurations were recorded for 24 hours in 12 replicates.

Data analysis

After completing the experiments, we analysed the obtained videos to determine: (i) movement initiation time (time from the introduction to the first activity, i.e. initiation of locomotion or burrowing), (ii) locomotion distance and (iii) mean burrowing level [%]. Every minute, we estimated the level of bivalve burrowing (using a 5-level percentage scale: 0, 25, 50, 75 and 100%) based on the length of the part of the shell below the substratum surface to the total shell length (according to Poznańska-Ka-kareko et al. 2021). Mean burrowing level was calculated according to the formula:

$$MB = \sum_{i=1}^{4} \left[25\%^{*} it_{i} \right] / \sum_{i=0}^{4} t_{i}$$
(1)

where: i – burrowing level: 5 steps ranging from 0 (totally exposed on the surface) to 4 (fully burrowed); t_i – time spent by the mussel at burrowing level i.

The list of all comparisons between the variants of Experiment 1 is presented in Table 3. As the data strongly violated normality and homoscedasticity assumptions (tested with Shapiro-Wilk and Levene tests, respectively), we used non-parametric Mann-Whitney U tests to compare mussel behaviour in experimental treatments, except for the comparisons among F-F/C2 variants of Experiment 1, where the same individuals were tested several times. For those, we used Wilcoxon signed rank tests for paired samples. In Experiment 2, we compared unionid behaviour in *Dreissena* sp. presence treatments to control treatments using Mann-Whitney tests to check unionid responses to the presence of biofoulers (chemical cues from biofoulers only). We applied a sequential Bonferroni correction for multiple comparisons (within each variable and species). However, we decided to report and interpret results both with and without the correction, as it is commonly considered as overly conservative with a large number of comparisons (Moran 2003). Statistical analyses were carried out using SPSS 29.0 (IBM Inc.).

Results

Experiment 1: Unionid responses to direct *D. r. bugensis* and *D. polymorpha* fouling and presence of fouled unionids

There was no significant effect of *Dreissena* spp. fouling on *S. woodiana* movement initiation time and locomotion (Fig. 2a, e). Mean burrowing level of cleaned *S. woodiana* (variant F/C1) was reduced compared to the control individuals (NF) but increased again after a longer exposure (F/C2) (Fig. 2g, Table 4).

For *U. tumidus*, we noted differences in movement initiation time: fouled mussels (F) started to move later compared to control non-fouled individuals (NF). When the fouled *U. tumidus* (F) were cleaned (becoming F/C1), they hastened their movement initiation time up to the level exhibited by the control individuals (NF). Moreover, cleaned *U. tumidus* (CwF) exposed in the company of fouled individuals started to move later (general activity) compared to *U. tumidus* kept in the absence of dreissenids (F/C1) and did not move horizontally at all (Fig. 2b, f, Table 4). *Unio tumidus* burrowed less when fouled (F) compared to the control non-fouled individuals (NF), and this effect persisted after cleaning (F/C1) (Fig. 2h, Table 4).

Table 4. Unionid responses to direct *D. r. bugensis* and *D. polymorpha* fouling and presence of fouled unionids in Experiment 1. Variant NF: mussels collected in the field without any trace of fouling on their shells, hereafter referred to as non-fouled mussels; variant F: mussels collected as fouled by *Dreissena* spp. and tested first time with their own fouling (fouled mussels); variant F/C1: F mussels, cleaned and tested after two days in the presence of non-fouled individuals (cleaned mussels); variant F/C2: F/C1 mussels tested on the next day (for the third time); variant CwF: mussels collected as fouled, cleaned two days before testing, and tested in the presence of fouled individuals. Variants NF vs F and F vs CwF were compared with Mann-Whitney U tests; variants F-F/C2 were compared to each other using Wilcoxon signed rank tests for paired data. Statistically significant differences are marked with asterisks and those that are still significant with the sequential Bonferroni correction are marked in bold font. Z – test statistic; P – statistical significance. Underlying data are available in Suppl. material 1.

Variable	Canfor	S. w	oodiana	U. tumidus			
variable	Comg	urations		Z	Р	Z	Р
Movement initiation time	Non-fouled (control, NF)	vs.	Fouled (F)	-0.69	0.488	-2.42	0.015*
	Non-fouled (control, NF)		Cleaned (F/C1)	-0.55	0.583	-1.21	0.225
	Fouled (F)		Cleaned (F/C1)	-0.16	0.875	-2.98	0.003*
	Cleaned (F/C1)		Cleaned (F/C2)	-0.16	0.875	-1.14	0.255
	Cleaned (CwF)		Cleaned (F/C1)	-0.40	0.686	-2.02	0.043*
Locomotion distance	Non-fouled (control, NF)	vs.	Fouled (F)	-0.79	0.429	-0.04	0.970
	Non-fouled (control, NF)		Cleaned (F/C1)	-0.41	0.684	-0.72	0.470
	Fouled (F)		Cleaned (F/C1)	-0.52	0.600	-0.34	0.735
	Cleaned (F/C1)		Cleaned (F/C2)	-0.85	0.398	-0.42	0.674
	Cleaned (CwF)		Cleaned (F/C1)	-0.41	0.684	-2.44	0.015*
Mean burrowing level	Non-fouled (control, NF)	vs.	Fouled (F)	-0.43	0.665	-3.12	0.002*
	Non-fouled (control, NF)		Cleaned (F/C1)	-2.54	0.011*	-2.94	0.003*
	Fouled (F)		Cleaned (F/C1)	-1.18	0.239	-0.63	0.530
	Cleaned (F/C1)		Cleaned (F/C2)	-2.04	0.041*	-1.88	0.060
	Cleaned (CwF)		Cleaned (F/C1)	-1.79	0.073	-0.17	0.862

Neither species showed significantly different movement initiation times or locomotion parameters exclusively due to passing time (comparison of F/C1 vs F/C2).

Experiment 2: Unionid responses to waterborne cues of *D. r. bugensis* and *D. polymorpha*

Sinanodonta woodiana showed a higher mean burrowing level in the presence of *D. r. bugensis* compared to the control (Fig. 3, Table 5). Neither the presence of *D. r. bugensis* nor *D. polymorpha* affected the locomotion and burrowing of *U. tumidus* (Fig. 3, Table 5).

Discussion

Consistently with our first hypothesis, dreissenid fouling affected the behaviour of unionid mussels. *Sinanodonta woodiana* responded only with changed burrowing level. In Experiment 1, we observed shallower burrowing of *S. woodiana* immediately after fouling removal, but, over time, burrowing returned to the level exhibited by the control, non-fouled mussels. On the other hand, in Experiment 2, *S. woodiana* burrowed deeper in the presence of quagga mussels compared to the control. This might be a defensive response of *S. woodiana* to



Figure 2. Unionid responses to the direct dreissenid fouling and presence of fouled unionids in Experiment 1: **a**, **b** movement initiation time [min] **c**, **d** locomotion distance [cm] **e**, **f** mean burrowing level [%]. Asterisks mark significant differences between the variants (ns – non-significant). Boxplots present medians (horizontal lines), 1st and 3rd quartiles (top and lower boxes, respectively), 1.5*interquartile range (whiskers) and outliers (circles). Variant NF: mussels collected in the field without any trace of fouling on their shells, hereafter referred to as non-fouled mussels; variant F: mussels collected as fouled by *Dreissena* spp. and tested first time with their own fouling (fouled mussels); variant F/C1: F mussels, cleaned and tested after two days in the presence of non-fouled individuals (cleaned mussels); variant F/C2: F/C1 mussels tested on the next day (for the third time); variant CwF: mussels collected as fouled, cleaned two days before testing, and tested in the presence of foulid individuals.

the presence of dreissenids, consisting in increased burrowing. It is worth noticing that a similar, though marginally non-significant tendency for increased burrowing was observed in *S. woodiana* exposed to fouled unionids (variants CwF vs F/C1, Fig. 2, Table 4).



Figure 3. Unionid responses to *D. r. bugensis* and *D. polymorpha* waterborne cues in Experiment 2. Asterisks mark significant differences between the variants (ns – no significant). Boxplots present medians (horizontal lines), 1st and 3rd quartiles (boxes), 1.5*interquartile range (whiskers) and outliers (circles).

The pattern observed for *S. woodiana* in Experiment 1 can be explained by contrasting effects of fouling (mechanical obstacle to efficient burrowing) and defensive responses to *Dreissena* spp. presence (stimulating burrowing). For fouled individuals, burrowing was more difficult, but they kept trying to defend themselves from fouling, resulting in a similar level as that showed by the control mussels.

¥7 · 11					odiana	U. tumidus		
Variable	Conngurations			z	Р	Z	Р	
Movement initiation time	control	vs.	D. polymorpha presence	-0.37	0.712	-0.60	0.545	
			D. r. bugensis presence	-0.17	0.862	-0.90	0.369	
Locomotion distance	notion distance control vs. <i>D. polymorpha</i> presence		-1.12	0.264	-0.60	0.551		
			D. r. bugensis presence	-1.11	0.266	-0.47	0.636	
Mean burrowing level control vs. <i>D. polymorpha</i> presence		D. polymorpha presence	-1.10	0.273	-0.30	0.762		
			D. r. bugensis presence	-2.14	0.033*	-0.98	0.327	

Table 5. Unionid responses to *D. r. bugensis* and *D. polymorpha* waterborne cues in Experiment 2: compared to their behaviour on the control (pairwise Mann-Whitney U test). Statistically significant differences are marked with asterisks (note that they do not pass the Bonferroni correction for multiple comparisons). z - test statistic; P - statistical significance. Underlying data are available in Suppl. material 1.

Immediately after the fouling removal, with no dreissenids present in the environment (variant F/C1, no need for anti-fouling defence), they burrowed more shallowly due to recently experienced problems related to fouling (e.g. exhaustion), but, with time (the next experimental round, F/C2), their burrowing returned to the control level. Nevertheless, this effect of fouling persisting after cleaning, though short-timed, suggests that the impact of dreissenids on their hosts was not purely mechanical, but also affected their condition.

Increased burrowing is a natural defence mechanism (Saloom and Duncan 2005) and may indicate an attempt to avoid fouling mussels, which, once attached, could have a more negative impact on their host condition than when acting only indirectly by their presence nearby (Sousa et al. 2011). Mussels are known to sense the presence of predators (Reimer and Harms-Ringdahl 2001; Meira et al. 2024) and parasites (Selbach and Mouritsen 2020) from chemical cues in the environment, as well as detect alarm signals from damaged conspecifics (Leonard et al. 1999), which results in the induction of various defence mechanisms, such as increased burrowing (Griffiths and Richardson 2006). The occurrence of a defence mechanism in S. woodiana may give this species an advantage in unfavourable conditions, such as the presence of biofouling Dreissena spp. The deeper the mussel burrows, the less exposed its shell is to the fouling dreissenids, which directly affects the degree of fouling, as shown by Urbańska et al. (2019). Moreover, existing fouling can also be reduced by the burrowing of the host unionid, as dreissenids are known to detach from unsuitable (e.g. burrowed) substrata and move in search of a better attachment site (Dzierżyńska-Białończyk et al. 2018b; Balogh et al. 2024). Dzierżyńska-Białończyk et al. (2018a) and Balogh et al. (2024) have shown that burrowed S. woodiana (but not the native unionid species, including U. tumidus) were less fouled by dreissenids than individuals exposed on the surface, confirming a defensive potential of this behaviour.

In Experiment 2, *S. woodiana* responded significantly only to the presence of quagga mussels, which confirmed that unionids can detect and respond to chemical cues of other bivalves in the environment (our fourth hypothesis). However, it should be noted that a similar tendency was also apparent in the presence of zebra mussels, thus the support for the hypothesis that unionid responses to fouling depended on the fouler species remains weak. Anyway, it is possible that *S. woodiana* is more familiar with quagga mussels, which are more abundant in Lake Balaton, although this ratio may vary in different parts of the lake (Balogh et al. 2008; Balogh et al. 2024). Moreover, quagga mussels are less associated with their

substratum (D'Hont et al. 2021) and easier to detach due to their weaker adhesion strength (Balogh et al. 2019), thus burrowing can be a more effective defence mechanism against them compared to more strongly and more permanently attached zebra mussels.

In Experiment 1, fouled *U. tumidus* showed a delayed movement initiation time and shallower burrowing compared to the control, non-fouled individuals. After the fouling removal, the movement initiation time of *U. tumidus* (variant F/C1) returned to the control level, but the burrowing remained weakened. Therefore, changes in the behaviour of *U. tumidus* were driven by the mechanical effect of fouling present on their shells, as well as by the impaired condition of fouled mussels. This supported our third hypothesis, as the fouling effect persisted after the fouling removal.

We did not observe any effects of direct fouling on the locomotion, in contrast to Van Appledorn and Bach (2007) working on North American unionids Ligumia nasuta Say, 1817 and Anodonta grandis Say, 1829. However, in accordance with our fourth hypothesis, U. tumidus responded to the presence of other fouled individuals by stopping any horizontal movements and delayed movement initiation (here: burrowing start, as locomotion did not occur at all) (CwF vs F/C1). Theoretically, this difference might be an artefact resulting from the fact that mussels from F/C1 were tested in the experimental arena for the second time, whereas for those from CwF it was the first experimental round. However, the comparison between animals tested repeatedly in consecutive experimental rounds under the same conditions (F/C1 vs F/C2) showed no significant differences and symptoms of exhaustion or habituation due to repeated testing, thus this explanation seems unlikely. On the other hand, the observed responses of U. tumidus to the presence of fouled individuals may represent a general reaction to stressed (here: fouled) conspecifics secreting some infochemicals indicating stressful environmental conditions. Definitely, U. tumidus did not respond to direct cues from dreissenids, as shown by Experiment 2.

All changes in the behaviour of *U. tumidus* induced by fouling or nearby presence of fouled individuals seem negative, exposing them to a number of environmental threats, including predation, parasites, dislodgement by water movements, desiccation during droughts, and further fouling by dreissenids (Bowers et al. 2005; Lymbery et al. 2021).

Our study showed that the behaviour of the native U. tumidus in the presence of fouling Dreissena spp. mussels was modified to a higher extent than that of the invasive S. woodiana. Moreover, the responses of S. woodiana appeared to have defensive and preventive effects against dreissenid fouling, whereas the behaviour of U. tumidus seemed to be impaired compared to their normal (control) activity. Thus, despite a similar relatively moderate level of fouling of both species in our study, dreissenids presence had stronger negative effects on U. tumidus. Due to large body size of S. woodiana and its weaker burrowing (Poznańska-Kakareko et al. 2021), more dreissenids, especially zebra mussels, can attach to and persistently stay on their surface than to smaller native mussels. Therefore, S. woodiana can act as a reservoir for dreissenids colonizing the native species, with the native species suffering more severe negative consequences of fouling. This suggests yet another potential effect of invasive dreissenids on native communities: their presence can influence interactions between native and invasive species of Unionidae, promoting the latter. On the other hand, S. woodiana can provide suitable substratum for dreissenid development. Moreover, we have shown that, beside parasites (McElwain 2019) and

predators (Meira et al. 2024), dreissenids can constitute another driver of apparent competition between invasive and native species, when the invader poses a threat to the native organism by increasing the abundance and availability of biofoulers. This effect is likely to be most pronounced in locations where shells of unionid mussels constitute the primary source of hard substratum available to dreissenids, such as soft lake bottoms, or dam reservoirs and river pools with limited flows and accumulated sediments, missing rocks and artificial embankments (Dzierżyńska-Białończyk et al., 2018a; Balogh et al. 2024). Nevertheless, such places are common in the environment and are frequently used by unionids. Therefore, dreissenid mussels can extend their distribution ranges as they are able to use their shells as attachment sites.

It should be noted that our research was conducted using mussels collected from a single location in Lake Balaton. This allowed us to eliminate potential confounding effects related to different conditions experienced by experimental animals before their sampling. We checked the situation taking place within the same natural community, living under the same conditions. However, we must admit that conducting the same research using mussels from other regions of the world may result in different results. To better understand the influence of dreissenids on Unionidae, it would be useful to conduct similar comparative studies with mussels from other lakes or rivers.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

KW: Conceptualisation, Methodology, Resources, Investigation, Formal analysis, Visualisation, Writing – Original draft, Writing – Review and Editing; CB: Conceptualisation, Methodology, Resources, Visualisation, Investigation, Writing – Review and Editing, Funding Acquisition; JK: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data Curation, Visualization, Writing – Review and Editing; DS: Methodology, Resources, Investigation, Writing – Review and Editing; ŁJ: Resources, Investigation, Writing – Review and Editing; MPK: Conceptualization, Methodology, Investigation, Validation, Visualization, Formal analysis, Supervision, Project administration, Writing – Review and Editing.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Dataset

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Research Article

Public preferences for edible invasive alien marine species -The Atlantic blue crab in southern Italy

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Abstract

Since 2014, the provision of the aquatic ecosystem services has been gradually affected due to the biological invasion of Callinectes sapidus (Rathbun, 1896, Crustacea, Decapoda, Portunidae), commonly known as Atlantic blue crab, across several lagoon-like locations in Italy. In addition, this serious aquatic invasive species, native of North American coasts, has already inflicted economic damage of about EUR 100 million to the Italian fishing and farming communities over the past year. To counter their severe and rapid spread, the Italian Government has encouraged the fishing communities to catch as many as possible and commercially exploit them for human consumption in an attempt to manage their expansion. Since there is an ongoing promotion for the consumption of blue crab by forging novel food businesses in Italy, this paper aims to predict the public preferences and their willingness to pay (WTP) towards this biological invader. For this purpose, a discrete choice experiment approach is used, by means of a multinomial logit model (MNL) and latent class model (LCM). The social field survey involves a representative sample of 440 valid respondents in Apulia Region, southern Italy. The descriptive statistics results reveal that 67.50% of the local citizens interviewed know about the blue crab invasion, while 29.09% of them have already consumed this seafood species. In addition, the MNL results show that the most appreciated attributes of the blue crabs by respondents are freshness and large size. Further, the LCM findings reveal two representative classes of Apulian consumers; the first group of citizens (70.9% of total respondents) expresses positive appreciation for consuming blue crabs, while the second group (29.1% of total respondents) is not willing to pay a premium price regarding this potential commercial activity. Furthermore, the econometric results show that the average value that Apulian's (i.e. those belonging to Class/Group I of respondents) WTP for blue crabs' consumption is about EUR 18 per kilogram. In this regard, this research has public and private implications and may reasonably promote the commercial exploitation of blue crabs, enhancing the reduction of its population density through human consumption as a promised management control strategy and forging a novel profitable business mainly for local small-scale artisanal fisheries.

Key words: Alien species consumption, aquatic ecosystem services, *Callinectes sapidus*, coastal biological invasions, discrete choice experiment, econometric analysis, social perception



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Introduction

Human shipping activities provide wide-ranging economic benefits (Balzani et al. 2022), while also driving biological invaders outside their native range, potentially leading to biodiversity, social and economic losses (Cardone et al. 2022; Demetriou et al. 2023), undermining human well-being and inducing significant economic management costs in recipient communities (Kourantidou et al. 2021a). This is especially true for aquatic invasive species, which also present a serious potential food competitor to native community fish (Ogorelec et al. 2022), threatening the structure and stability of the marine ecosystems (DeRoy et al. 2022). Climate change, in terms of high temperatures and low precipitation, is also contributing to the impact on the taxonomic and functional diversity of marine species communities (van Deurs et al. 2021; Souza et al. 2023; Saifi et al. 2024). In this direction, the Callinectes sapidus (Rathbun, 1896, Crustacea, Decapoda, Portunidae), known as the Atlantic blue crab (Fig. 1a), an aquatic invasive species, native to North American coasts (Sabelli 2023), is a relevant case of this biological coastal invasion phenomenon and is considered amongst the most aggressive of the marine invasive groups (Swart et al. 2018). In the context of international trade, blue crabs have been introduced by commercial large vessels that use ballast water for weight adjustment, controlling buoyancy and stability. By pumping unfiltered water during their transoceanic crossing, these vessels can accidentally load invasive crustaceans, such Callinectes sapidus and then discharge them into a new non-infected seabed, such the Euro-Mediterranean Basin, at the end of their voyage, thus initiating the blue crab's invasion process (Nehring 2011). As such, the first observation of blue crab's species occurred in the French Atlantic coasts in 1900, in the Mediterranean Sea in the 50s, in Egypt at the end of the 19th century, in Italy (Apulia Region, southern Italy, Fig. 1b) in 2014 and in Tunisia (Gulf of Gabes, Tunisia's east cost in the Mediterranean Sea) in 2015 (Mancinelli et al. 2017). On the one hand, blue crabs are recognised as voracious biological invaders due to their: (i) omnivore and feeding flexibility (Johnson et al. 2014), (ii) exponential increase and various abundance across spatial and temporal aquatic systems and seasons (Mancinelli et al. 2017), (iii) rapid invasion in non-native habitats (Clavero et al. 2022), (iv) severe economic, social and environmental impacts (Marchessaux et al. 2023) and (v) strength of interactions with other marine species (Prado et al. 2024). On the other hand, these aquatic creatures are generally appreciated as healthy seafood species by consumers due to their high nutritional values (i.e. excellent source of proteins and minerals) and their organoleptic quality (Nanda et al. 2021) in terms of sweetness, tender meat, unique flavour and delicious taste. Against this background, there is increasing interest in assessing the benefits and costs of ecosystems services in real-world situations (Courtois et al. 2014).

In this sense, the reduction of its population through commercial exploitation for human consumption in Italy, as has already been developed in several countries, such as Egypt (Abdel-Razek et al. 2016), Greece (Kevrekidis et al. 2013), Turkey (Ayas and Ozogul 2011; Harlıoğlu et al. 2018), Tunisia (Ennouri et al. 2021) and USA (Sharov et al. 2003), is proposed as one of the best comprehensive management controls of this threat (Marchessaux et al. 2023). Similarly, this invasion may yield changes in the human well-being of Apulian fishermen and may be considered as a potential source of revenues, enhancing the livelihoods of local artisanal small-scale fisheries, as mentioned above and detailed in Fig. 2.



Figure 1. a an example of blue crabs catches in Lake Lesina – Lagoon – north of Apulia Region (southern Italy, Fig. 1b). The blue crab is an omnivore having: (*i*) an average life span in the wild from 1 to 3 years; (*ii*) a size of 4 inches (10 cm) long, 9 inches (23 cm) wide and (*iii*) a weight of 1 to 2 pounds (453 to 907 g) **b** the Italian geographical position of Lake Lesina. Fig. 1b also highlights of the hotspots (in red) where *Callinectes sapidus* has been observed in the study area. Source: Ludovica Nardelli (2023), co-author. Appendix 1 gives an overview of the marine fishing sector in Apulia Region, the study area.

Callinectes sapidus invasion considered as a:							
Biological threat, inducing negative impacts such as:	Commercial interest, inducing positive impacts such as:						
 Ecological losses in terms of: Habitat modification Biddiversity loss 	Development of artisanal small-scale fisheries						
• Decrease of the quality of the marine ecosystem services	Industrialisation of blue crabs species						
 Socio-economic losses for human activities in terms of: Impacts on fisheries (i.e., damage to fishing nets, increased hard work; physical damage to fishermen; decrease of commercially shellfish revenues; degradation of the image of coasta lareas and products; decrease in people's welfare) 	Development of tourism activities: Integration of blue crabs on the restaurants menus						
 Impacts on tourism industry (i.e., decrease in tourism revenues; damage to coastal residents, fishing and aquaculture tourism and water-based recreation) 	Improvement of local food diet, in which the blue crabs present a high nutritional value						
 Impacts on catches (i.e., physical damage to fish; mortality of catches in the net; decrease of local species density) 	\clubsuit Increase in local employement to manage/catch the blue crabs						
 Additional costs of private management & medical treatments 	Increase in public & private management programs towards biological coastal invasions						

Figure 2. Overview of the impacts of *Callinectes sapidus*, based on the scientific paper of Marchessaux et al. (2023). In this regard, changes in marine ecosystem services and human well-being due to edible invasive alien marine species give rise to assess public's preferences about propensity purchase and willingness to pay a premium price for its consumption that would be considered as a promised sustainable control measure.

Meanwhile, not all invasive alien marine species can cause negative damage (Minasidis et al. 2023) and their perception may not be perceived similarly by all social public groups. Therefore, assessing the propensity purchase and willingness to pay a premium price for its consumption as a control measure constitutes a crucial component of the economic analysis of invasive species that is used when government choices are considered for controlling biological threats (Emerton and Howard 2008). Moreover, Apulian consumers as contributors may pay incremental charges towards this coastal biological invasion. Consequently, their preferences are crucial in the decision-making process to systematically manage such phenomena in terms of effectiveness and cost efficiency. Without this previous consideration, there is likely to be a risk of wasteful and imprudent resource allocation (Frem et al. 2021).

In this direction, this study focused on Apulia Region, south-eastern Italy (Fig. 1b) and was carried out to elicit consumers' preferences for Apulian blue crabs and to estimate their WTP, enhancing the purchase and, consequently, the consumption of blue crabs in their regional markets. Additionally, this research identifies consumers market segments through the: (*i*) specific attributes (see section Methodology) of this marine invasive species, (*ii*) propensity and attitudes of purchase, as well as (*iii*) socio-economic and demographic characteristics of respondents, within an econometric analysis of biotic invasive framework. Specifically, this study addressed three interrelated research questions: (i) what are the consumers' profiles for Apulian blue crabs; (ii) what are their preferences for this marine invasive species and (iii) what are their WTP for consuming Atlantic blue crab?

However, to address these research questions, we opted for an econometric Discrete Choice Experiment (DCE) approach (Petrontino et al. 2022, 2023a, 2023b), to provide insights into biological invasion policy decision-making. In this regard, the originality of the present paper is dual. First, to the best of our knowledge, this is the first econometric and social study on one of the most recent coastal biological invaders in Italy, where the need for reduction options management is of primary governance importance. In this sense, the Italian Government has recently allocated EUR 2.9 million (MASAF 2023, see Appendix 2) to tackle the aggressive invasion of the Atlantic blue crabs, which are threatening the fishing industry, mainly relating to clams, mussels and oysters¹, in Italy. These seafood attributes sustain the Apulia economy and are served in their many traditional dishes. Second, the present paper enhances the peer-reviewed literature that uses DCE to elicit social perception regarding the coastal invasion by non-indigenous species. In recent years, there have been a few studies that have used DCE to give insights into invasive species management. For example, Bougherara et al. (2022) estimated spatially differentiated preferences for the management of primrose willow (Ludwigia grandiflora), an invasive weed spreading in a French regional park. They use a DCE to estimate people's WTP to control the invasion of this

¹ Interspecific relationships (predation: predator-prey, trophic competition) play a crucial role in shaping marine ecosystems. With respect to predation, the latter involves one species (the predator, here: the blue crab) consuming another (the prey, here: e.g. clams, mussels and oysters), affecting their population dynamics and community structure, regulating prey populations, influencing their abundance patterns and shaping marine community dynamics. Regarding the trophic competition, the latter occurs when species compete for the same resources (i.e. food, habitat). As such, the blue crab may compete with other species (i.e. other crabs, fish) for similar prey, affecting habitat selection and ecological niches.

species in different areas of the regional park. Frem et al. (2021) used a DCE in Italy to map residents' preferences heterogeneity and their WTP to regenerate the olive landscape that has been severely affected by Xylella fastidiosa, an invasive bacterium. Bekele et al. (2018) used a DCE to assess the economic value of dry land ecosystem services that had been degenerated by the invasive Prosopis spp., in Ethiopia and Kenya. Malpica-Cruz et al. (2017) conducted a DCE to assess the potential economic impacts of the Lionfish invasion in the Mexican Caribbean, in which lionfish-control fees were acceptable to some, but not to all recreational users. Additionally, Zeilinger et al. (2014) address the importance of consumer feeding preference amongst resource choices, inducing critical implications for ecological risk assessment and invasion biology. Finally, Rai and Scarbourgh (2013) designed a DCE to estimate WTP to mitigate damages caused by invasive plant species in a rural community of Nepal. Compared to the number of DCE studies on management of invasive species, there have been more DCE studies on the consumption of seafood species using a DCE approach. For example, Sigurdsson et al. (2023) investigated how different combinations of signals (i.e. certificates/tags; health/sustainability) impact consumers' choice and WTP for fish fillets. Zheng et al. (2023) estimated United States consumers' willingness to pay for genetically modified salmon and examined heterogeneity in preferences across levels of consumer perceptions, knowledge and attitudes. Rodriguez-Salvador and Dopico (2023) investigated consumers' preferences for the origin and traceability of fish products. Risius et al. (2019) elicited target groups for fish from sustainable aquaculture and their preferences for different countries of origin. These previous studies are based on native fish or seafood products rather than on alien aquatic species. In this respect, the results of this research triggered private and public implications, forging a potential novel profitable fishery business and raising social awareness with respect to an edible marine invasive species like Callinectes sapidus.

Methods

In this paper, we implemented a DCE approach that is used in valuation of marine ecosystem services and marine food products, but less so for edible alien marine species. Thus, our paper contributes to the scarce literature on revealing people's preferences for edible invasive alien marine species and the socio-economic characteristics and other factors that drive their WTP to consume such species. In line with similar studies, this approach involved five major components, such as: (i) Identification, description of attributes and designation of their levels, (ii) Development of an experimental design and construction of choice set, (iii) development of a social-choice survey questionnaire and data collection, (iv) Econometric estimation models regarding the measurement of consumers' preferences by the means of a Multinomial Logit Model (MNL), development of cluster profiles using Latent Classes Model (LCM) and estimation of WTP in each of these cluster profiles, as well as (v) Interpretation of results for policy analysis or decision support. The WTP estimates of consumers reflected the hypothetical change in the utility (i.e. sense of satisfaction) or acceptability or unacceptability (Börger et al. 2014; Malpica-Cruz et al. 2017) associated with the consumption of invasive alien species, such Callinectes sapidus as a contribution to its management.

Identification of attributes and designation of levels

As a first component of the DCE, we first based the identification of attributes and levels on the empirical literature related to consumer purchasing behaviour towards fish and seafood products (Carlucci et al. 2015), including country of origin, product methods, preserving methods, product innovation, packaging, eco-labelling etc. Hence, we used an approach involving a focus group of experts to select the considered attributes and design their correspondent levels. From an invasion management perspective, understanding consumer preferences for preservation methods can help to elaborate value-added products within a supply chain where, where the cold chain or the direct refrigeration of freshly caught products is guaranteed. Consequently, it may have implications for those directly involved in fishing and preserving the product before placing it on the market to more effectively and, at the same time, sustainably manage the alien species. Similarly, the place of purchase may be of interest for the same reasons that may prompt different actors in the distribution chain to take an interest in blue crab management, including through awareness-raising campaigns. Subsequently, we retained six characteristics with three levels each as illustrated in Table 1.

The first category included three levels of seafood species (i.e. blue crab, spider crab and pink shrimp). The selection between these species might help to make a comparison between invader and non-invader common marine seafood attributes. In other words, we looked to compare the blue crab with two products that were

		Level 1	Level 2	Level 3
	ies	Blue crab	Spider crab	Pink shrimp
	seafood spec		E	alle -
	gn b	Fresh/Chilled	Frozen	Canned
	Preservi metho			
		Small	Medium	Large
Attributes	Size			
	പ ന	Italy	Apulia region	Abroad
	Fishin origii	e contraction de la contractio	En and a second and a second and a second a se	
		Fishermen or Direct purchase	Fishing shops	Supermarkets or Hypermarkets
	Place of purchase			
	Price	EUR 10 per 1 kg	EUR 17 per 1 kg	EUR 23 per 1 kg

Table 1. Attributes and levels with symbols or pictograms selected to explore the hypothetical social perception and willingness to pay towards *Callinectes sapidus* invasion in Apulia Region.

Source: our elaboration.

close to it in terms of recognisability (i.e. shrimp as a common consumed shellfish and spider crab as a similar species already known by most Italian consumers). The second category involved three levels of preserving/storage methods (i.e. fresh/ chilled, frozen and canned) for each designated seafood species. The preference between these preserving/storage methods might enhance relevance for fish stakeholders marketing several seafood products. The third category concerned the three levels of sizes (i.e. small, medium and large) for each identified seafood species. The selection between these sizes for each involved seafood species might address the preferences of Apulia consumers towards their most favourite size of marine fish species. The fourth category involved three levels for fishing/capture origin (i.e. Apulia, Italy and abroad), for each identified seafood species. The choice between these fishing origins might highlight which origin will be the main influencing preference for Apulia consumers for the concerned seafood species. The fifth category entailed three types of location for the purchase of the three seafood species (i.e. fishermen/direct purchase, fish shops and supermarkets/hypermarkets). The decision between these places for purchase might indicate which location might be considered as a driver or a barrier for the consumption of the concerned seafood species. The last attribute was related to the premium price that Apulian consumers would pay for their consumption of 1 kg of each considered seafood species (i.e. EUR 10, EUR 17 and EUR 23 per kg versus the status quo or EUR 0). We based this range of prices on a price survey on the Italian goods exchange system. Hence, this set of selling prices was considered to make the hypothetical market more compatible with prices that respondents see daily in stores. Furthermore, this category was considered as a discrete variable in the DCE, leading to estimate the consumers' willingness to pay for the consumption of blue crabs and to assess the monetary trade-offs that consumers make for each category and level considered in this study. We also illustrated all attributes by symbols or pictograms, supporting the respondents in their choice process (De Ayala et al. 2012) or providing a stimulus in which they might easily select a choice set (Zoderer et al. 2015).

Development of an experimental design and construction of choice sets

The experimental design followed a standardised procedure of conducting a pilot survey. This served to set up preliminary coefficients for the final experimental design and, based on the sign obtained in the respective levels, alternatives with implausible combinations were eliminated. Examples include alternatives that simultaneously contained very low prices with levels of the other attributes presumably leading to increased utility; or, conversely, high prices with levels of the other attributes presumably leading to disutility. After selecting the attributes and their levels (Table 1), we employed a full factorial experimental design, generating a high number of combinations 729 (36) and making it logistically unfeasible and impractical to administer all this quantum of choice sets to one respondent. Hence, we opted for a D-efficient fractional factorial design (Bush 2013) with an efficiency score of 0.85, using the code of package AlgDesign on R (Suppl. material 1). To prevent complexity, minimise confusion, survey costs and cognitive fatigue for respondents during the survey (Frem et al. 2021), as well as to improve efficiency (Zheng et al. 2023), the set of 60 scenarios were distributed into three blocks including five choice sets (i.e. known as "Options") each. In each choice set, we included four options. In this line, having three instead of one or two choice

options, in addition to the status quo, may also have positive implications, such as greater realism. However, including too many options may lead to respondent fatigue and potential uncertainty in preferences as stressed above. Consequently, we found that using three options was acceptable, given the extensive experimental design in terms of the large number of attributes and levels. As such, by offering three distinct options, the choice sets more closely reflect the real-world scenarios that participants may face when they make decisions about the topic being studied. Fig. 3 illustrates an example of a choice set used in our DCE approach. Each respondent had the opportunity to select one option amongst the four hypothetical options (A, B, C and D as an opt-out option), leading to a maximisation of his/ her total utility for the consumption of blue crabs, assuming that this utility was a function of a selection in seafood species attributes and other determinants (i.e. size, place of purchase, fishing origin and price as a budget constraint).

Development of a social-choice survey questionnaire and data collection

We developed a structured questionnaire (Suppl. material 2) for data collection between May and October 2023 in Apulia Region. We tested the questionnaire with a sample size of 25 respondents, randomly selected from Apulia residents, taking into consideration their age and gender. The aim of this pilot survey was to test the effectiveness of the levels for each attribute, eliminate all irrelevant questions, estimate the time needed for face-to-face interviews and to guarantee that respondents fully understood the questionnaire. The language of the questionnaire was Italian and the survey was limited to Apulia residents.

The questionnaire was divided into three sections. The first section concerned the purchasing habits and propensities of Apulia citizens. This section included attitudinal questions dealing with individuals' general attitudes towards the purchase habits and propensities of food, fish products/seafood and their knowledge and consumption of the blue crabs, along with 13 questions, such as: "Do you personally take care of food purchasing for your family? (i.e. Yes; No) (Q1); How often do you shop for food? (i.e. once a day; more than once a week; once a week; more than



Figure 3. Example of a choice set used in our Discrete Choice Experimental (DCE) approach.

once a month; once a month; less than once a month; never) (Q2); Are you allergic to shellfish? (i.e. Yes; No) (Q3); How often do you consume shellfish? (i.e. always; often; sometimes; rarely; never) (Q4); Where do you usually buy products such as shellfish or related fish products? (i.e. direct sales (fisherman); fish shops; super-markets; hypermarkets and shopping malls; other) (Q5); When buying seafood products, how much attention (i.e. not at all; little; quite a lot; a lot; very much) do you pay to the following characteristics? (i.e. place of purchase; commercial seafood species; origin of the product; price) (Q6); Do you know about the blue crab (i.e. Yes; No) (Q7); Have you ever consumed it? (i.e. Yes; No) (Q8); If you have never consumed it, can you give a reason? (Q9); How often do you buy blue crab? (i.e. always; often; sometimes; rarely; never) (Q10); Where did you buy it? (it is possible to select more than one answer) (i.e. direct sales (fisherman); local fisheries markets; supermarkets; hypermarkets; other) (Q11); Where did you most commonly consume it? (i.e. restaurant; events; home; other) (Q12); On a scale of 1 to 10, report your product satisfaction index about the consumption of blue crab (Q13).

At the end of this section, interviewees were informed about the current invasion of blue crab in Italy and its negative (i.e. a biological threat impacting the provision of ecosystem services and inducing socio-economic losses for human activities) and positive (i.e. potential source of revenues) implications on the fishery sector in Apulia. Two relevant images on blue crabs supported this section. The second section concerned the preferences of Apulia citizens for the consumption of blue crabs. In this section, we asked the respondent to make choices as described above (Fig. 3). We provided here five purchase simulations (i.e. choice sets) where the respondent could choose between three options (options A, B and C) which differ in the selected attributes and levels (Table 1). In addition to the three available purchase options, there was a no-purchase option (option D or opt-out) that he/she could choose if none of the three options offered satisfied him/her. At the end of each choice set, respondents were asked about their choice certainty, using a scale from 1 (absolutely uncertain) to 5 (absolutely certain) as depicted in Table 2, in which the overall mean of all respondents was equal to 3.83, indicating

Table 2. Respondents' certainty level of their choice using a scale from 1 (absolutely uncertain) to 5 (absolutely certain).

Choice set N°	Option N°	Mean	Std. Deviation	Min	Max
1	1	3.79	0.679	2	5
1	2	3.85	0.762	2	5
1	3	3.95	0.571	2	5
2	1	3.73	0.741	2	5
2	2	3.83	0.757	2	5
2	3	3.86	0.587	2	5
3	1	3.79	0.690	2	5
3	2	3.67	0.757	2	5
3	3	3.87	0.527	3	5
4	1	3.80	0.622	2	5
4	2	3.89	0.596	2	5
4	3	3.97	0.450	3	5
5	1	3.76	0.606	2	5
5	2	3.93	0.663	2	5
5	3	3.90	0.520	3	5

	Year	Male	Female	Total		Year	Male	Female	Total
		Number (Aj	pulia Region)				Number (A	pulia Region)	
	18-44	606,237	587,116	1,193,353		18-44	63	68	131
	45-64	576,840	609,472	1,186,312		45-64	73	86	159
_	≥ 65	413,081	517,356	930,437		≥ 65	71	79	150
atio	Total	1,596,158	1,713,944	3,310,102	ple	Total	207	233	440
luqo		In % (Apu	ılia Region)		San		In % (Apı	ılia Region)	·
	Year	Male	Female	Total		Year	Male	Female	Total
	18-44	38%	34%	36%		18-44	30%	29%	30%
	45-64	36%	36%	36%		45-64	35%	37%	36%
	≥ 65	26%	30%	28%	1	≥ 65	34%	34%	34%

Table 3. Sample of Apulian participants field social survey used in our Discrete Choice Experiment.

an overall "enough certainty" of their choices. The third section aimed to collect data about the key socio-economic characteristics (i.e. gender, age, residence, civil status, family composition, level of education, work position, work sector and annual household income) that could contribute to their decision process.

The final survey involved 440 respondents in the study area, considering the Apulia population age and gender distribution, in which the sample was in a similar range to the main statistics of Apulia population (Istituto Nazionale di Statistica - ISTAT 2023) as reported in Table 3. For this purpose, we interviewed by block at least 60 respondents randomly distributed between the sexes (at least 30 females and 30 males) and ages (at least 25 males and females for each of the following age classes: 18 to 44, 45 to 64 and greater than 65 years old). For the statistical and econometric analysis, we only retained the respondents who declared that they were the main person responsible for shopping for food for household consumption.

Econometric estimation models: MNL and LCM

The DCE approach is based on the random utility maximisation framework and the theory of product attribute values (Lancaster 1966), in which a consumer's perception utility ("U") of terrestrial, marine and aquatic ecosystems or seafood products, such blue crabs, is generated from their attributes. The consumers' utility ("U") consists of deterministic (observable) and probabilistic (non-observable) components (Louviere et al. 2000). Using this theoretical foundation, we assumed that each attribute included in this study constituted one component of the blue crabs' utility which can be expressed as follows:

$$Uni = V_{ni} + \varepsilon_{ni} \tag{Eq. 1}$$

where: "n" is the users (i.e. respondents/consumers), "i" is the alternatives (choice sets, Fig. 3), " V_{ii} " is a function of observable attributes of the blue crabs, known as the deterministic component of "U" and " ε_{ni} is a function of the non-observable characteristics of the blue crab's seafood product and respondent-level variation in unknown perceptions, preferences or attitudes considered as the stochastic part of "U" and treated as random error.

In line with similar studies, we also assumed an additive utility function linear of the observed attributes levels (Table 1). Based on this, "*Uni*" becomes:

$$U_{ni} = \alpha + \beta_1 x_{1n} + \beta_2 x_{2n} + \dots + \beta_m x_{mni} + \varepsilon_{ni}$$
(Eq. 2)

where: " α " is a constant term; " x_{ni} " are the attributes of the alternatives ("*i*") for each respondent ("*n*") and " β " are the coefficients of the attributes of the options; " β " also reveals the preference weight for each attribute level, as well as trade-off monetary values; " β " represents the importance of the attribute level to the utility function that respondents/consumers give to an option.

When dealing with two or more options, the respondent will thus select the option associated with the highest utility (i.e. benefit or satisfaction). Thus, the probability that the nth respondent chooses the ith option from a choice set becomes:

$$P_{ni} = \operatorname{Prob}(U_{ni} > U_{nj}) \ \forall j \neq i = \operatorname{Prob}(V_{ni} + \varepsilon_{ni} > V_{ni} + \varepsilon_{ni}) = \forall j \neq i = \operatorname{Prob}(\varepsilon_{ni} - \varepsilon_{ni} < V_{ni} - V_{ni}) \ \forall j \neq i$$
(Eq. 3)

To estimate " β " and their corresponding standard errors for each level of the six selected attributes (Table 1), we initially used the Multinomial Logit Model (MNL) which provided the basis for the analysis of DCE as innovated by McFadden (1974) for exploring choice behaviour in relation to the elements that describe the option. However, MNL has two limitations. First, the equal measurement of utility and the assumption of preference homogeneity amongst all respondents. In other words, MNL captures only the mean of preferences across the studied population. To counter these limitations, Random Parameters Model (RPL, known also as Mixed Logit Model) or LCM are used to account for and identify heterogeneity in respondents' choice preferences. RPL assumes that each respondent can have his/her own unique set of parameters, whereas LCM assumes that the studied population is divided into a finite number of classes or segments, each with their own set of parameters. Lastly, RPL offers a more nuanced view of individual-level variation, whereas LCM provides a more straightforward interpretation by identifying distinct groups (Vermunt 2003). Consequently and based on the specific needs of this research, we opted for the LCM because it can segment consumers into classes, based on their choice patterns. We assumed that interviewed consumers were heterogeneous in their attributes and preferences towards the consumption of blue crabs, that may differ according to some socio-economics characteristics and habits, as well as propensity for purchase and that blue crabs control fees might vary between consumers, leading to the adoption of targeted public communication and information at a specific group that will more likely induce a greater management impact towards the concerned biological invader rather more broadly than the communication policy (Malpica-Cruz et al. 2017). In this sense, the probability that a respondent will choose an alternative *i* is a function that includes the attribute levels of alternative *i* and the attribute levels of all other alternatives presented in Table 2 and becomes as follows:

$$Prob(choice = i) = \frac{exp^{V(\beta, Xi)}}{\sum_{i} exp^{V(\beta, Xi)}}$$
(Eq. 4)

where: $V(\beta, x_i)$ is the observed component of the utility function for alternative *i* and *j* is a set of alternatives.

Regarding the LCM, this model assumes that the studied population is divided into different unobserved/latent classes with regards to the attributes and levels and disentangles the probabilistic presence of any discontinuity in the heterogeneity of respondents, thus enabling them to cluster into homogeneous classes or segments, so that preferences are identical within the segment, but differ between them. As such, the LCM offers the opportunity to identify population heterogeneity and better understand the target respondents, leading to appropriate management interventions directed towards encouraging consumption of blue crab by particular groups of consumers.

In this direction, we applied LCM as a statistical clustering procedure (Weller et al. 2020) with two selected subclasses of respondents within the sample, relying on rigorous statistical tests (He and Fan 2019) and assuring the best representation of the target blue crabs consumer's market. For this purpose, we implemented a model fit statistics information criterion (Table 4), in which the most optimum fit improvement (i.e. optimum number of classes) is determined by the following criteria: maximum log-likelihood (IC), minimum Bayesian Information Criteria (BIC) and minimum adjusted Akaike Information Criteria (AIC), indicating the greatest amount of variation and using the fewest possible independent variables (Emiliano et al. 2014; Petrontino et al. 2022). As such, we applied these criteria to several classes of our respondents to select a reasonable number of clusters/groups with a good assurance regarding the stability, sensitivity and specificity of data as depicted in Table 3. Furthermore, the choice probability (Eq. 4) within a class *q* becomes as follows:

$$Prob(choice = i/\beta_q) = \frac{exp^{V(\beta_q, X)}}{\sum_{i} exp^{V(\beta_q, X_i)}}$$
(Eq. 5)

With respect to WTP, we estimated the WTP that reflected the average price a respondent would pay for blue crabs' consumption for each of the two selected classes or groups of respondents (Nylund-Gibson and Hart 2014). We also performed a Wald procedure according to the Krinsky-Robb method, used with 500 draws. In this regard, we estimated the WTP by using the following equation:

$$WTPk = -\frac{E(\beta k)}{\beta(price)}$$
(Eq. 6)

where k_s are the attributes, WTP_k is the expected WTP for k, $E(\beta_k)$ is the estimate of the coefficient for attribute k and β (price) is the price coefficient.

	Multinomial Logit	2-Class	3-Class	4-Class	5-Class	6-Class
Log-likelihood	-2050	-1989	-1948	-1920	-1898	-1886
Adjusted Akaike Information Criteria (AIC)	4124	4028	3972	3942	3924	3926
AIC/N	2.27	2.22	2.19	2.17	2.16	2.16
Bayesian Information Criteria (BIC)	4190.046	4165.596	4181.146	4222.696	4276.246	4349.796
Adj BIC	4190.084	4165.753	4181.504	4223.34	4277.259	4351.265
Average classes probabilities	100%	29.1%	9.1%	27.7%	36.1%	35.7%
		70.9%	26.5%	40.3%	35.1%	2.1%
			64.4%	22.6%	9.9%	9.7%
				9.4%	11.4%	16.1%
					7.5%	29.6%
						6.8%

Table 4. Selecting a class solution: Model fit statistics information criteria.

Results

Statistical description of respondents

This section includes basic statistical results from the first and third sections of the questionnaire (Suppl. material 1), addressing the purchasing habits and propensities of Apulia residents towards fish products, their knowledge of the blue crabs, as well as their socio-economic characteristics. In this direction, Table 5 reveals that most respondents (82.5%) were personally in charge of food purchase more than once a month, while 2% of them had a shellfish allergy. A total of 57% of the participants in this social survey purchased seafood products at the fish shops, followed by supermarkets (20%), while 8% of them purchased directly from fishermen. Further, the price of seafood products was the most determining factor in their purchase decision, followed in succession by the preservation method, the place of purchase, the origin of fishing and the type of the seafood species. Regarding their knowledge of blue crabs, 67.50% of them were familiar with this product, while 29.09% of them had already consumed it.

In addition, 43.64% of them confirmed their ignorance about this seafood category as a key reason for non-consumption, while a few of them (9.32%) declared their difficulty in finding this product on the local fish market as a reason of non-consumption. Regarding their socio-economic profiles, on average, respondents were middle-aged (53.7 years old), female (53%) and widely distributed amongst their levels of education (primary school: 2%, secondary school: 22%, high school: 38%, university: 37%). The average length of the studies undertaken by the respondents was 13.6 years, while the average family size was nearly three members. In terms of the total annual gross family income, it was distributed as follows: 23.6% (less than EUR 25,000), 55.5% between EUR 25,000 and 50,000) and 20.9% (greater than EUR 50,000).

Econometric estimates of multinomial logit model (MNL) and latent class model (LCM)

The MNL estimates are reported in Table 6 and revealed that many coefficients of the concerned attributes ("seafood species – blue crab and spider crab; preserving method – canned; size – small; fishing origin – abroad") presented negative signs for the price and were highly significant at the notable level of 1%. On the contrary, the MNL findings showed that the opt-out coefficient ("no purchase" or option D) was equal to -0.63 and highly significant, indicating that the Apulian consumers would opt to purchase seafood species products. However, this econometric model only allowed us to elicit the mean preference contribution and might hide the individual variations of preferences amongst the samples, which might present different preferences as addressed below by the LCM model. In addition, Table 5 also revealed that the later model achieved better values in terms of the log-likelihood function AIC and BIC compared to the MNL model. With respect to the later model, the sample was divided into two classes of Apulian consumers, based on their perceptions and expectations towards non-indigenous aquatic species. According to the iteration performed for different number of classes, AIC improves as the classes increase, but it led to an excessive complication of the model. Therefore, BIC was used as a discriminant in the choice of the classes. Regarding class 1, Table 6, in coherence with the MNL results, depicted also negative signs and high statistical significance at the 1% level for the concerned attributes, implying a high level of influence on

Variable description	Category	Mean/%	SD	Min	Max
Are you personally in charge of food purchases?	Yes	82.5%			
Frequency of food purchase	1: Once a day; 2: More than once a week; 3: Once a week; 4: More than once a month; 5: Once a month; 6: Less than once a month; 7: Never	5.57	1.15	2	7
Do you have a shellfish allergy?	Yes	2%			
Frequency of shellfish consumption	1: Always; 2: Often; 3: Sometimes; 4: Rarely; 5: Never	2.952	0.73	1	5
Place of purchase of shellfish	Fisherman	8.0%			
Place of purchase of shellfish	Fish shop	57.0%			
Place of purchase of shellfish	Supermarket	20.0%			
Place of purchase of shellfish Hypermarket	Hypermarket	15.0%			
Place of purchase of shellfish	Other	0.0%			
Attention to product characteristics:	Place of purchase	3.27	0.89	1	5
Attention to product characteristics	Conservation method	3.42	0.79	1	5
Attention to product characteristics	Commercial species	3.24	0.77	1	5
Attention to product characteristics	Origin	3.23	0.86	1	5
Attention to product characteristics	Price	3.60	0.69	2	5
Do you know the blue crab?	Yes	67.50%			
Do you consume blue crab?	Yes	29.09%			
Reason for non-consumption	Dislike	5.91%			
Reason for non-consumption:	Allergy/intolerance	1.36%			
Reason for non-consumption	Cost	1.36%			
Reason for non-consumption	No-knowledge	43.64%			
Reason for non-consumption	difficulty of retrieval	9.32%			
How often do you buy blue crab?	1: Always; 2: Often; 3: Sometimes; 4: Rarely; 5: Never	1.37	0.67	1	5
Place of purchase of blue crab	Direct sale (fishermen)	12.27%			
Place of purchase of blue crab	Local fisheries markets	9.32%			
Place of purchase of blue crab	Supermarkets	2.50%			
Place of purchase of blue crab	Hypermarkets	5.00%			
Place of purchase of blue crab	Other	70.91%			
Place of consumption	Restaurant	7.50%			
Place of consumption	Events	3.64%			
Place of consumption	At home	17.27%			
Place of consumption	Other	71.59%			
Male	%	47%			
Female	%	53%			
Family members	Number	3.12	1.11	1	5
Education level	No education	0%			
Education level	Primary school	2%			
Education level	Secondary school	22%			
Education level	High school	38%			
Education level	University	37%			
Education (Total years of study)	Number	13.580	3.994	5	18
Gross household income	< EUR 25 000	23.6%			
Gross household income	≥ 25 000 EUR ≤ 50 000	55.5%			
Gross household income	> FUR 50 000	20.9%			

Table 5. A summary of all the descriptive statistics of the sample.

the consumer decisions. On the contrary, the preservation method ("fresh/chilled") and size ("large") coefficients were positive and highly significant for MNL and both groups of LCM models, as anticipated, indicating that these attributes provided Apulian consumers with great utility regarding, at least, the physical appearance of the seafood species. In addition, the signs for the attributes regarding the fishing origin ("Apulia Region") and the place of purchase ("fishermen" and "supermarket or hypermarket") were positive in general, reflecting the concerns of Apulian residents to keep or create local jobs and support local economies. They may also be more

	Multinomial Logit Model (MNL)		Latent Class Model (LCM)				
A •1	100%		Class 1 (29.1%)		Class 2 (70.9%)		
Attribute	Coefficients						
	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value	
Seafood species ("Blue crab")	-0.279**	0.029	-1.931***	0.000	0.529***	0.000	
Seafood species ("Spider crab")	-0.724***	0.000	-2.032***	0.000	-0.129	0.405	
Preserving method ("Fresh")	1.217***	0.000	2.498***	0.000	1.128***	0.000	
Preserving method ("Canned")	-0.804***	0.001	0.225	0.637	-1.130***	0.000	
Size ("Large")	0.729***	0.000	1.212**	0.01	0.497***	0.000	
Size ("Small")	-0.447***	0.004	-0.722*	0.093	-0.388***	0.003	
Fishing origin ("Apulia Region")	0.195	0.187	0.208	0.5715	-0.142	0.277	
Fishing origin ("Abroad")	-0.189	0.195	-0.047	0.891	-0.213*	0.092	
Place of purchase ("Fishermen")	0.270*	0.080	0.114	0.725	0.274**	0.015	
Place of purchase ("Supermarket/hypermarket")	0.129	0.322	0.136	0.737	0.636***	0.004	
Price	-0.055***	0.000	-0.060**	0.031	-0.029***	0.000	
Opt-out	-0.633***	0.009	0.207	0.706	-0.597**	0.013	
Model statistics	· · · ·				· · · · ·		
Criteria	MNL		LCM				
Log Likelihood	-2050		-1989				
Adjusted Akaike Information Criteria	4124		4028				
AIC/N	2.27		2.22				
Bayesian Information Criterion	4190.046		4165.753				
Number of observations	18	15	1815				
Number of variables	1	2	25				

Table 6. Multinomial Logit Model (MNL) and Latent Class Model (LCM) results.

Note: ****, ***, * ==> Significance at 1%, 5%, 10% level, respectively. The seafood species (pink shrimp) was the baseline in the econometric estimation results.

aware about the local fishing practices than those which are practised by overseas fisheries, as well as providing them with appreciated services in the supermarket or hypermarket in which a set of diversified seafood attributes is present. Furthermore, both classes of respondents had a negative price coefficient, but class 2 presented a higher intensity and highly significant coefficient, indicating that a small price variation might induce them not to purchase the product. In addition, the attribute related to the place of purchase ("fishermen") was appreciated by the respondents of the entire sample, since its coefficient had a positive value, but with different significance levels amongst the samples, thus verifying the hypothesis of heterogeneous consumer preferences for seafood species consumption. Moreover, the MNL results indicate a clear preference hierarchy: consumers prefer pink shrimp over blue crab and they like blue crab over spider crab. The preference for blue crab over spider crab indicates that, while blue crab may be less favoured than pink shrimp (used as a well-known widely consumed species), it is still viewed more favourably than spider crab (used to represent a quite similar alien species). The indirect implication of this hierarchy resides in the possibility that policy managers might implement regulations that promote responsible harvesting practices over other species and effective educational campaigns. Making consumers aware of the ecological impacts of blue crab invasions and the potential threats they pose to local ecosystems can influence their purchasing decisions. MNL per se does not tell us the characteristics of respondents but LCM does. It can be useful to address the mentioned educational campaigns based on the two classes characteristics.

Estimates of willingness to pay (WTP)

The WTPs (in EUR) estimation are reported in Table 7 and were obtained from positive and statistically significant LCM coefficients estimates (Table 4). As a result, the respondents of class 1 were not willing to pay a premium price for blue crabs and to consume as many as possible, in an attempt to manage the expansion of this invasive species in Italy. This result indicated the lack of sufficient awareness and information within this class of consumers about the potential benefits of the commercial exploitation of this aquatic invasive species as depicted in Fig. 2. On the contrary, respondents of the second class were willing to pay EUR 18.01 per kg on average for blue crabs' consumption, suggesting that the preferences of a representative part of Apulian residents would consume it, contributing to the control of the concerned non-indigenous aquatic species. Further, the respondents of this group were willing to pay more for the freshness attribute of seafood products, in which the related average WTP was estimated at EUR 33.06 per kg, indicating the relevance for fish stakeholders marketing several seafood products. Furthermore, the WTPs were positive, but relatively less for the size ("large") and place of purchase ("fishermen" or "supermarket/hypermarket"), compared to blue crabs' species and freshness attributes, suggesting these attributes to be considered as drivers for the consumption of the concerned seafood species. Finally, the preserving method ("fresh/chilled") presented the highest average WTP in both classes, in which this was estimated at EUR 46.38 and 33.06 per kg for respondents of class 1 and class 2, respectively, providing Apulian consumers with high utility or organoleptic satisfaction.

Discussion

Interpretation and comparison

The findings detailed in the Results section provide an understanding towards the perceptions and expectations of Apulian consumers, constituting one of the market drivers for any successful novel food product, such blue crabs (Kaimakoudi et al. 2013). In this regard, we applied a DCE approach to investigate the influence of the type of seafood species (i.e. blue crab, spider crab and pink shrimp), preserving/ storage methods (i.e. fresh/chilled, frozen and canned), size (i.e. small, medium and large), fishing/capture origin (i.e. Apulia, Italy and abroad), as well as the selling price (i.e. EUR 10, EUR 17 and EUR 23 per kg versus the status quo or EUR 0) for each selected seafood species which may influence the consumers' preferences.

	0 17 7						
Class	V:-11-	WTP	Standard	Z	Prob.	95% Confidence Interval	
	variable		Error		z >Z*		
1	Preserving method ("Fresh/chilled")	46.3819	190.7	0.24	0.8078	-327.38	420.147
	Size ("Large")	20.42	47.7826	0.43	0.6691	-73.232	114.072
2	Seafood species ("Blue crab")	18.0131	12.0209	1.5	0.134	-5.5474	41.5736
	Preserving method ("Fresh/chilled")	33.0611	16.5714	2	0.046	0.5818	65.5404
	Size ("Large")	16.1509	8.6523	1.87	0.0619	-0.8073	33.1091
	Place of purchase ("Fishermen")	8.38602	5.77782	1.45	0.1467	-2.9383	19.7103
	Place of purchase ("Supermarket or hypermarket")	10.0112	6.29548	1.59	0.1118	-2.3277	22.3501

 Table 7. Willingness to pay (WTP) estimates.

As a result, we found that most Apulian inhabitants (around 70% of the whole sample) were not opposed to introduce the blue crabs into their nutritional diet/ food system and were willing to pay a positive average amount of EUR 18.01 per kg for blue crabs, prompting fishermen and retailers to adjust their pricing strategies accordingly in line with consumer expectations and maximize revenues. In fact, we observed that the retail market price at which a blue crab was sold to consumers, oscillated between EUR 8 to 12 per Kg at most supermarkets across Apulia region during 2023. This price is normally influenced by several factors including production costs, competition, demand and market conditions. As such, the blue crabs, which have a market value of around €80 a kilogram in the United States and Asia, appear not to be able presently to fetch much on the Italian market. However, the findings in terms of WTP differ by social class membership and type of attributes. In fact, the Apulian inhabitants gain higher utility for the preserving method ("fresh/chilled") and size ("large") attributes, but, for the place of purchase ("direct purchase or supermarket/hypermarket"), respondents of class 2 expressed relatively lower WTPs, indicating that the freshness attribute appears here to be a determinant driver for Apulian inhabitants' consumption of seafood species, such blue crabs. Moreover, the MNL results indicate a clear preference hierarchy: consumers prefer pink shrimp over blue crab and they like blue crab over spider crab. The preference for blue crab over spider crab indicates that, while blue crab may be less favoured than pink shrimp (used as a well-known widely consumed species), it is still viewed more favourably than spider crab (used as a quite similar alien species). The indirect implication of this hierarchy resides in the possibility that policy managers might implement regulations that promote responsible harvesting practices over other species and effective educational campaigns. Making consumers aware of the ecological impacts of blue crab invasions and the potential threats they pose to local ecosystems can influence their purchasing decisions. MNL per se does not tell us the characteristics of respondents, but LCM does. It can be useful to address the mentioned educational campaigns, based on the two classes characteristics. Furthermore, the findings illustrate significant differences between the two classes of the studied population for most of the variables related to the purchasing habits and propensities of Apulia residents towards fish products, their knowledge of the blue crabs, as well as their socio-economic profile. With respect to "Class 1", this respondents' segment had higher food purchase frequencies, but relatively lower shellfish consumption. In addition, this class had a higher percentage of respondents with low income and a higher number of household members. Moreover, in this group, the knowledge of blue crab was higher and we observed the largest number of people who cited taste and the presence of allergies as reasons for non-consumption. Regarding "Class 2", these respondents consumed shellfish in general and blue crabs more frequently than the first group. The attention to the price of products was higher and the highest percentage of purchases was directly from the fisherman. In this segment, respondents had higher educational level and male respondents were older than in Class 1 as observed in Table 8. Consequently, our findings are consistent with a recent EU consumer study on habits regarding fishery and aquaculture products in Italy (European Union 2021), as well as with previous similar studies in the Euro-Mediterranean Basin. For example, Minasidis et al. (2023) stated that Greek consumers would buy and consume non-indigenous fish species, in which the freshness was ranked as the most important factor for the purchase of this marine species. Moutopoulos et al. (2022) observed that consumers' attitudes towards the consumption

of Pearl oyster (*Pinctada imbricata radiata*) differ according to their socio-economic profile, in which highly-educated consumers were more willing to purchase and consume this kind of marine species. Additionally, Cerveira et al. (2022) found that Portuguese consumers were willing to consume another edible aquatic invasive species, the Weakfish *Cynosian regalis* (Bloch & Schneider, 1801). In addition, Marchessaux et al. (2023) reported that 58% (33% in Italy) have already consumed and appreciated both blue crab species. However, our findings are in line with Petrontino et al. (2022) who also reflected on the importance of the geographic or fishing origin as the seafood consumption driver. Lastly, Grover et al. (2021) found that Australian households were willing to pay \$AUD 37 per year for 5 years for the management of native and invasive species in coastal waters off the east coast of Tasmania.

Table 8. Comparison between the two classes of the studied population, for the purchasing habits and propensities towards fish products,their knowledge of the blue crabs, as well as their socio-economic profile.

¥7 · 11			Mean or %	
variable Category		Class 1	Class 2	P [*]
Frequency of food purchase	1: Once a day; 2: More than once a week; 3: Once a week; 4: More than once a month; 5: Once a month; 6: Less than once a month; 7: Never	6.097	5.858	< 0.001
Frequency of shellfish consumption	1: Always; 2: Often; 3: Sometimes; 4: Rarely; 5: Never	2.922	3.015	< 0.001
Attention to product characteristics	Place of purchase	3.32	3.288	0.177
Attention to product characteristics	Conservation method	3.495	3.446	0.018
Attention to product characteristics	Commercial species	3.223	3.285	0.003
Attention to product characteristics	Origin	3.194	3.254	0.008
Attention to product characteristics	Price	3.544	3.608	< 0.001
Do you know the blue crab?	Yes	1.427	1.258	< 0.001
Do you consume blue crab?	Yes	1.748	1.665	< 0.001
Reason for non-consumption	Dislike	0.087	0.054	< 0.001
Reason for non-consumption	Allergy/intolerance	0.039	0.004	< 0.001
Reason for non-consumption	Price	0.01	0.008	0.393
Reason for non-consumption	Lack of knowledge	0.417	0.435	0.184
Reason for non-consumption	Difficulty of retrieval	0.078	0.077	0.914
How often do you buy blue crabs?	1: Always; 2: Often; 3: Sometimes; 4: Rarely; 5: Never	1.272	1.446	< 0.001
Blue crab's satisfaction (index of evaluation)	Scale of 1 to 10	7.37	7.573	0.017
Age	Year	52.214	54.931	< 0.001
Gender	Female	0.398	0.442	< 0.001
Family members	Number	3.146	3.004	< 0.001
Education	Total number of studies	13.718	13.746	0.784
Gross household income	< EUR 25 000	0.272	0.223	< 0.001
Gross household income	≥ 25 000 EUR ≤ 50 000	0.534	0.573	0.002
Gross household income	> EUR 50 000	0.194	0.204	0.354
Place of purchase of blue crab	Direct sale (fishermen)	2%	11%	(baseline)
Place of purchase of blue crab	Local fisheries markets	2%	7%	< 0.001
Place of purchase of blue crab	Supermarkets	1%	2%	0.505
Place of purchase of blue crab	Hypermarkets	1%	4%	< 0.001
Place of purchase of blue crab	Other	21%	47%	< 0.001
Place of consumption	Restaurant	2%	6%	(baseline)
Place of consumption	Events	1%	3%	0.011
Place of consumption	At home	4%	14%	0.127
Place of consumption	Other	21%	48%	< 0.001
Education level	Primary school	1%	1%	(baseline)
Education level	Secondary school	6%	16%	< 0.001
Education level	High school	11%	28%	< 0.001
Education level	University	11%	27%	< 0.001
Limitations and future research directions

The first limitation of this research includes its regional level coverage. Future DCE studies should counter this issue by selecting a national representative sample to explore potential insights into Italian regional differences and communities in attitudes and propensity to purchase and consume blue crabs. A second limitation is related to the use of two criteria (age and gender) to the sampling method adopted. However, follow-up studies should include the annual revenues of participants in the survey and their residence, reflecting their culture and traditions (Sacchettini et al. 2021) and inducing a better representation of Italian consumers towards their social perception and WTPs to control the Callinectes sapidus invasion in Italy. A third limitation of this study considers the "general public" as participants/respondents in our DCE approach, excluding other key fish stakeholders that may present a greater preferences utility for consumption of blue crabs. As such, we may suggest an extension on examining the interest utility amongst other groups of respondents to cover the preferences of tourists and ethnicity (Sayeed et al. 2022) and of local fish entrepreneurs or other groups of stakeholders (i.e. fishermen, seafood species processors, retailers, consumer organisations) for whom the human consumption of this biological invader would become a sustainable effective management tool, inducing positive impacts on their income and enhancing their financial performance. Here, it would be beneficial to conduct further research, based on a cost-benefit analysis (Courtois 2004; Courtois et al. 2014; Rajmis et al. 2016; Frem et al. 2022) allowing us to: (i) assess the impacts on commercial shellfish fisheries, (ii) estimate the costs of this blue crab invasion, (iii) justify its public management expenditure (Falk-Petersen and Armstrong 2013) and identify the maximum economic yield, preventing potential losses from overharvesting practice. Additionally, bio-economic modelling (McDermott et al. 2013; Varble and Secchi 2013; Benjamin and McDermott 2018) should be addressed in the future to explore how this potential commercial exploitation (Kourantidou and Kaiser 2021b) of the blue crab would really help to manage its invasiveness, taking into consideration two possible scenarios: (i) minimising its population pressure at the lowest possible level, while protecting adult females and critical nursery habitats like underwater grasses which are crucial for future crab numbers and (ii) allowing a sustainable level of catches for fishermen (i.e. to stabilise a certain level of biomass of the invasive alien species, maintaining a balance where catches do not exceed sustainable levels) across several Italian seas. In this line, Italian governmental efforts are being made to manage the blue crab invasion through resource allocation to fishermen. Allowing a commercial fishery for human consumption will increase fishermen's income, but might not ensure sustainable exploitation (Nardelli et al. 2024). According to EBFM (2010), the strengths of blue crabs' exploitation include: (i) Economic value (i.e. blue crabs may provide livelihood and income for many fishermen. The fishery supports local fishermen's' income and a potentially significant processing sector for crabmeat production, contributing to the local economy); (ii) Recreational fishery for blue crabs also supports a major recreational fishery, providing potential opportunities for recreational fishermen to enjoy crabbing activities; and (iii) Market demand; i.e. blue crabs appear to be in demand in both commercial and recreational sectors, with a variety of markets for fresh, frozen or processed crab. However, the blue crab exploitation also has weaknesses in terms of: (i) Vulnerability to overexploitation (i.e. blue crabs are

susceptible to overexploitation due to their economic importance, which can lead to population declines if not managed sustainably); (ii) Habitat degradation (i.e. fishing pressure can impact living habitats for blue crabs, such as salt marshes, leading to alterations in trophic interactions and potential habitat loss as stressed in the Introduction section); (iii) Environmental stressors (i.e. factors like climate change, habitat degradation and pollution can affect blue crab populations and their habitats, making them more vulnerable to exploitation) and (iv) competition with imports (i.e. globalisation of seafood markets has led to competition with imported crab products, affecting the market for local blue crabs and putting pressure on domestic fishermen). The blue crab exploitation may also include opportunities such as: (i) Sustainable management practices (i.e. implementing sustainable fisheries management practices can help ensure the long-term viability of blue crab populations and the fishery); (ii) Market diversification (i.e. exploring new markets and value-added or elaborated products can help diversify the market for blue crabs and assure further economic opportunities for fishermen); and (iv) Ecosystem health (i.e. blue crabs may play a significant role in the affected Italian lagoon ecosystem and their sustainable exploitation can contribute to ecosystem health and balance).

Furthermore, as the blue crab has usually been identified as a bioindicator organism of polychlorinated biphenyls, polycyclic aromatic hydrocarbons and methyl mercury (Ghaeni et al. 2015), as well as source pollutants for trace elements contamination (Cubedo et al. 2018; Salvat-Leal et al. 2020), we may also recommend the exploration of the acceptance of the adoption by respondents of the block-chain traceability system within the blue crabs value chain. This would mainly be applied for fishing activities and conservation methods as an innovative digital tracking tool for this kind of aquatic invader, which may influence positively or negatively, as well as significantly, their potential purchase decision. Lastly, the present choice experiment model could be enhanced by involving other specific blue crabs attributes related, but not limited to: (i) the level (greater or lesser) of blue crab's impacts on the ecosystem, on other economic sectors and on propagation in which the DCE may capture this crucial information related to invasive species management; (ii) the level or types of fisheries, such as small-scale units for development and industrialisation; (iii) the integration of blue crabs on to restaurants menus; (iv) the improvement of local food diet, in which the blue crabs present a high nutritional value, the social aspect in terms of creation or increase in local employment and public and private communication, research and management activities towards biological coastal invasion.

Conclusion

The present paper reveals the existence of two blue crab's consumer segments, reflecting a potential market for an edible marine invasive species. By capitalising on its exploitation opportunities as addressed above, stakeholders should work towards sustainable blue crab exploitation that benefits both the environment and Italian local economies. Thus, sustainable management practices, habitat conservation efforts and market strategies would be crucial to safeguarding the long-term health and sustainability of blue crab populations in the study area, in line with EU REG 1380/2013. In addition, the implemented DCE approach provides, in this paper, estimates through the estimation of WTPs that are useful in making private decision or public policy support. In this direction, one of the most significant findings of this study is that an important part of the Apulian inhabitants' sample (70%) expressed their interest towards the consumption of blue crabs and, consequently, to potential commercial exploitation of blue crabs as a novel food source. As such, this result provides a first good preliminary insight for fish entrepreneurs and restaurants to integrate this novel food into their shops and menus, respectively. In this direction, the development of this kind of novel food business requires raising public awareness through policy-makers and educational institutions and communication about its consumption benefits, to target mainly the segment of consumers who were not willing to pay a premium price towards the blue crabs' consumption in Italy. This could also probably lead to a change in their intentions and perceptions, making them more responsible and predisposed to buy edible aquatic invasive species. In addition, the adoption of a suitable targeted marketing strategy by the firms or fishery cooperatives involved in the catches of fish would reinforce the image of this aquatic invader, promoting its sustainable consumption in the near future.

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Additional information

Conflict of interest

Michel Frem was employed by Sinagri S.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical statement

The data gathered from the in-person survey was solely utilised for statistical analysis and the specific research project. According to Regulation (EU) 2016/679, personal data will not be shared with third parties or used for personal interests, whether one's own or others. The information obtained was solely utilised in a collective manner, ensuring the utmost anonymity of the participant. Additionally, respondents were asked for their consent at the start of the survey to take part in this research in line with national laws and institutional rules.

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Author contributions

Michel Frem: Writing – original draft, Writing – review & editing, Conceptualisation, Methodology, Formal data analysis, Supervision, Validation and Visualisation. Ludovica Nardelli: Writing – review & editing, Data collection. Alessandro Petrontino: Writing – review & editing, Conceptualisation, Methodology, Formal data analysis. Ståle Navrud: Writing – review & editing and Validation. Maria Antonietta Colonna: Writing – review & editing. Vincenzo Fucilli: Writing – review & editing, Funding acquisition, Supervision and Validation. Francesco Bozzo: Writing – review & editing, Funding acquisition and Validation.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Appendix 1

Marine fishing overview of the study area

The Apulian fleet has a strong social and economic dependence on artisanal fishing. According to the National Statistics Institute the fishing fleet in the study area is composed of 1629 vessels distributed amongst the seven maritime Compartments of this region: the Manfredonia Compartment possesses the highest number of boats (31.43%), followed by Gallipoli (22.28%), Bari (17.31%), Barletta (9.21%) and Brindisi (5.89%) in 2020. However, the Molfetta Compartment has the lowest number of boats (3.38%). The overall production of the Apulian fleet is around 7000 tonnes, of which 75.87% are captured through the otter trawling technique followed by fixed longlines (9.79%), anchored gillnets (4.00%), dredgers pulled by boats (2.50%) and purse seine (1.13%). In 2020, the catches per unit were equal to 4208 kg. With respect to the importance of the different fishing methods in Apulia, the significant volume of 5.2 tonnes relating to the "trawling with divergent" technique (75.87%) reflects the highly heterogeneous character of Apulia fishing. However, the two fishing techniques, "gillnets (drift) and beam trawling", are not practical in this Region. Furthermore, the "hand-line" technique is used in a very limited manner for catching fish in the study area. In addition, Apulia has a total tonnage of 18,500 GT and an engine power of 122,234 kW, of which the fishing technique with an otter trawl has the highest percentage in terms of tonnage (71.71%), followed by the techniques of: purse seine (12.71% in GT), fixed longlines (8.16% in GT), dredgers pulled by boats (4.41%) and anchored gillnets (2.45%). The average size of a boat in Apulia is 11.4 tonnes, compared to a national average of 14.2 tonnes in 2020.

Appendix 2

Italian financial aid to encounter the spread of the blue crabs: a summary

The rules governing the production and trade of fishery and aquaculture products marketed in Italy fall under EU's Common Market Organisation in Fishery and Aquaculture Products (CMO) Regulation, which is one of the pillars of EU's Common Fishery Policy. Consequently, the sale of the blue crab is currently not prevented by the CMO regulation, meaning that the consumption and even marketing of this crustaceous, not currently on the list of invasive alien species (IAS) of community interest, does not go against the EU's policy of managing the market for fishery and aquaculture products (European Union regulation 2013 available at https://eur-lex.europa.eu). Moreover, in the case that the blue crab is included in this list later, it would be subject to numerous restrictions under Article 7 of IAS EU Regulation and consequently, could not be placed on the market, stored, used or traded. However, another article of the IAS Regulation says that the commercial use of already established IAS may be temporarily permitted, but only as part of management measures aimed at their eradication, control or containment of the population. Meanwhile, to counter the spread of the blue crab species (Callinectes sapidus and Portunus segnis) throughout the Italian national territory and prevent the aggravation of the damage inflicted to the economy of the fishing sector, the Italian Ministry of Agriculture (*Ministro dell'agricoltura*, *della sovranità alimentare e delle foreste*, hereafter MASAF) released a decree law of 10 August 2023, in which article 10 authorised the expenditure of EUR 2,900,000.00 in favour of the aquaculture and fisheries companies that catch and dispose of the aquatic species mentioned above.

This Ministerial Decree (MASAF 2023) defined all eligible costs incurred for measures taken to catch and dispose of blue crabs, in particular: (1) costs for the catch (i.e. purchase of fishing gear specially used for catching blue crabs, such as pots, gillnets and cages, containment nets), (ii) costs for disposal (i.e. costs incurred in the disposal of blue crabs as waste at Italian establishments approved or registered under Reg. (EC) 1069/2009 in respect of animal by-products listed by the Ministry of Health - Directorate-General for Food Hygiene and Food Safety and Nutrition and transport costs of blue crabs to facilities authorised for disposal, such as forklift hire, cold storage hire, waste containers, plastic boxes, bins). Only expenditure incurred from 1 August 2023 until 31 October 2023 was eligible. However, the purchase of towed fishing gear was not eligible for the contribution. Moreover, the grant is provided in the form of a non-repayable contribution to the extent of 80% of the costs actually incurred in relation to the eligible interventions. Moreover, the contributions referred to in this Decree may be accumulated with any other State aid in relation to the same wholly or partly overlapping eligible costs, only where such accumulation does not lead to the highest aid intensity or aid amount applicable to the type of aid concerned being exceeded. In addition, the territorial competent Marine Authorities were assigned the tasks of control regarding the requirements previewed from the present Decree that can happen also after the distribution of the contribution. To the correct accomplishment of the controls, the Ministry puts at the disposal of the Maritime Authorities in a timely manner all the documentation produced by the applicants for the purposes of obtaining the contributions referred to in this Decree.

Supplementary material 1

Experimental design

Author: Michel Frem

Data type: xlsx

Explanation note: Statistical experimental design of the research: Discret choice experiment.

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Supplementary material 2

Surevy questionnaire

Author: Michel Frem

Data type: docx

Explanation note: Social survey used in this research based on the Discrete choice experiment approach.

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Research Article

Experimental evidence of negative agricultural impacts and effectiveness of mitigation strategies of invasive green iguanas (*Iguana iguana*) in Puerto Rico

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Abstract

Losses in crop yield due to invasive insects, weeds, pathogens, and herbivores cost trillions of dollars per year globally. To prevent further spread of invasive agricultural pest species, continuous monitoring and prevention are crucial. Once introduced, however, assessing the impact of an invasive pest on agricultural production and testing management strategies are essential. The green iguana (Iguana iguana), a globally widespread invasive herbivore, is considered a possible agricultural pest although no quantitative data on its impact are available. In this study, we evaluated the impact of the invasive green iguana on cucumber (Cucumis sativus, var. Dasher II) and lettuce (Lactuca sativa, var. Black-seeded Simpson) yield by testing the efficacy of two management strategies - Neem-based pesticide and mesh fencing - compared to open field cultivation in Puerto Rico. Mesh fencing led to 20% more growth and doubled cucumber yield compared to open field cultivation, while spraying Neem led to an 18% increase in plant growth but no effect on cucumber yield. We found no difference in lettuce growth or yield among treatment and control plots. This study supports categorizing the green iguana as an invasive agricultural pest species and demonstrates the reptile's potential to reduce crop yield. It also shows that Neem application at the manufacturer's suggested concentration is not an effective mitigation technique for reducing crop loss due to green iguana herbivory. Government agencies in regions where the green iguana has the potential to be introduced should consider the species a threat to food production when developing monitoring programs and drafting regulations.

Key words: Agricultural loss, biological invasion, cost of invasive species, exclusion experiment, invasive species in agriculture

Introduction

Invasive species are a threat to global agricultural production (Paini et al. 2016); researchers estimate that invasive weeds, insects, pathogens, and other organisms lead to annual losses of \$1.4 trillion USD (Pimentel et al. 2001, 2005; Zenni et al. 2021). The potential impact of invasive species on agricultural production is highest for developing nations (Paini et al. 2016) that may lack the economic means to mitigate or manage invaders. Within these countries, small farmers are at the



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highest risk due to their heavy reliance on their own food production as their main means for survival (Pratt et al. 2017). To support farmers and crop production, pest management plans are developed by policy makers to mitigate the detrimental impacts of invasive species on crop production (Stoddard et al. 2010; Ditomaso et al. 2017). A farmer's willingness to adopt mitigation measures is often tied to the recognition that a particular pest species is responsible for significant economic losses (Bajwa et al. 2019). Even when economic losses are realized, research on the effectiveness of different mitigation methods is often lacking.

Determining the extent of crop loss caused by a potential pest is critical for justifying prevention measures (Senar et al. 2016; Bajwa et al. 2019; García-Díaz et al. 2021). Exclusion studies are a useful tool in determining the severity of a pest species' impact on crop yield, as well as for testing management methods (Chouinard et al. 2017; Tollington et al. 2019). A study on cucurbits (e.g., cucumber, squash and melon) grown in high tunnels in Indiana (USA) sought to prevent pest beetles from accessing the plants (Ingwell and Kaplan 2019). Researchers tested three different mesh net sizes and found that intermediate nets were the optimal size for increasing yield. For some cucurbit varieties tested, yield was three times higher using the intermediate net size compared to no net, demonstrating the importance of testing management strategies to maximize crop yield. In addition to insect pests, exclusion netting has also been used to evaluate the impact of birds (Kuesel et al. 2019) and bats (Maas et al. 2019; Tollington et al. 2019) on crop yields. Though exclusion is widely used to protect crops, this strategy does not always increase crop yield (Maas et al. 2013). For example, a study on the coffee berry borer in Hawai'i assessed the impact of mesh netting on borer infestation levels, coffee quality, and coffee yield (Johnson et al. 2020). Researchers found higher borer densities in no-netting control plots but no differences in coffee quality or yield among treatments. Experiments to determine the effectiveness of management strategies are thus critical for providing useful management tools for the farming community.

Management recommendations aimed at reducing the negative impact of pest species often focus on controlling pest populations (García-Díaz et al. 2021) rather than mitigation strategies. In agriculture, population control (i.e., eradication or reduction in population size) for larger vertebrate species is difficult because pesticides cannot be used. Research on invasive vertebrates in the U.S. highlights the difficulties of implementing eradication and control measures (Witmer et al. 2007; Witmer and Fuller 2011). For example, eradication efforts targeting sheep in Hawai'i's Mauna Kea Forest Reserve have been unsuccessful despite the removal of 87,000 sheep by aerial hunting over a 75-year period (Hess and Jacobi 2011). Researchers and practitioners agree that developing management techniques beyond population reduction is necessary for future success (Witmer and Fuller 2011).

Testing invasive species management techniques on farms can have the two-fold benefit of providing policy makers with important information regarding the efficacy of management techniques while quantifying the economic impact needed to justify the development of pest management schemes. A good system to investigate this approach is the green iguana (*Iguana iguana*, Linnaeus, 1758), a widespread invasive species for which little information about its impact or management exists. The green iguana is native to Central and South America but has expanded its range most notably during the 1990's through the pet trade (Stephen et al. 2012). It can now be found on islands of the Pacific, the state of Florida, and the Greater Caribbean Region, among other places (Falcón et al. 2012; van den Burg et al. 2020; De Jesús Villanueva et al. 2021). This reptile is a generalist herbivore and can exist in a wide variety of vegetative communities (Bughardt and Rand 1982). Information on the diet of this species is limited to a handful of studies in its native and introduced ranges. In Mexico, gut content identification found mostly Ipomoea sp. (the sweet potato genus) and Tabebuia sp. (a woody tree genus) as part of its native diet (Lara-López and González-Romero 2002). In Puerto Rico, mangroves (Rhizofora mangle, Avicennia germinans), pond apple (Annona glabra), and the yellow flamboyant tree (Pelophorum pterocarpum) were identified through isotopic analysis and germination of seeds found in feces (Govender et al. 2012; Burgos-Rodríguez et al. 2016). In Fiji, anecdotal accounts of green iguana foraging in village food gardens have reported Ipomoea sp. and Dalo (an important root crop, Colocasia sp.) as diet items, although the authors believe that more evidence is needed to consider the species a threat to food production (Kern 2009; Van Veen 2011; Shah et al. 2020). Based on interviews with the farming communities in Puerto Rico, researchers identified more than 30 crop species consumed by green iguanas, with squash (Cucurbitaceae) and tomato (Solanaceae) crops being among the most consumed (De Jesús Villanueva et al. 2022).

The green iguana's impact on agriculture is often cited by researchers and wildlife professionals as negative (López-Ortiz et al. 2012; López-Torres et al. 2012), although research on the topic is only just emerging (Rodríguez Gómez et al. 2020; De Jesús Villanueva et al. 2022). In Puerto Rico, where the green iguana has been documented since 1964 (Rivero 1998; De Jesús Villanueva et al. 2021), the species is popularly considered an agricultural plague (ElNuevoDia.com 2009; López-Ortiz et al. 2012). Work by De Jesús Villanueva et al. (2022) found that farmers on the island manage this species to prevent crop loss. Hunting, physical barriers (e.g., nets or metal fencing), changes in crop choice, and chemical deterrents were among the management practices reported. The local Department of Natural Resources and the Environment has recommended the use of Neem oil (*Azadirachta indica*) as a repellent, together with the removal of eggs from nests (López-Ortiz et al. 2012; López-Ortiz 2013). None of these management practices or recommendations have been evaluated for effectiveness, leaving their utility up to the perceptions of the practitioners.

In this study, we sought to quantify the impact of the green iguana on agricultural production and to test the utility of currently employed management techniques. To determine if green iguana management on farms leads to increased crop yield, we used two agricultural crops reported as impacted by the green iguana in Puerto Rico, cucumbers (*Cucumis sativus*) and lettuce (*Lactuca sativa*), and two management techniques, mesh fences and Neem-based repellent (De Jesús Villanueva et al. 2022). We tested these management techniques at two agricultural experimental stations in Puerto Rico, where based on the findings of De Jesús Villanueva et al. (2022), we expected green iguana herbivory to significantly reduce crop yields. We compared crop yields in our experiment to observed yields prior to the presence of green iguanas on farms in Puerto Rico (Abrams et al. 1976).

Methods

Site description

We conducted experiments at two agricultural experimental stations (AES) on the Caribbean island of Puerto Rico. These AES are part of the University of Puerto Rico Mayagüez agricultural extension program and are in the towns of Juana Diaz (18.032318, -66.528910) and Gurabo (18.255926, -65.987933). We chose these two sites to conduct our experiments based on observations by field station agronomists of green iguana-related crop loss at each site and our own confirmation of the presence of green iguanas at each AES. To confirm the presence of green iguana on the two sites, we used visual encounter surveys (VES) at both field sites. The VES were repeated once a week for two months from June to August 2019. During the VES, three observers on average walked along each farm's fence line in a linear path adjacent to the experimental site for 200 m. Observations began at 0800 h and continued at a steady pace, stopping only to take note of the observations, until the 200 m length had been walked. Observations were made by eye, and binoculars were used to confirm observations when necessary. The climatic and soil conditions of the two sites were distinct from one another (see Suppl. materials for further details). The Juana Diaz Experimental Station is at an elevation of 0.0 Meters Above Mean Sea Level (MAMSL), and it is within the semi-arid climatic zone of the island (Goyal and Gonzalez 1989). The Gurabo Experimental Station is at an elevation of 52.0 MAMSL, and it is located within the moist climactic zone (Goyal and Gonzalez 1989).

Plant cultivation

To test how green iguanas may affect the cultivation and harvest of crops, we focused on two crops commonly grown in Puerto Rico: lettuce, *Lactuca sativa* (var. Black-seeded Simpson) and cucumber, *Cucumis sativus* (var. Dasher II). Lettuce and cucumber seedlings were purchased from local germination companies and transplanted into mulched plots covered in black plastic (4.6 m long by 1.5 m wide) at each AES. In each plot, 15 plants of either lettuce or cucumber were planted. Spacing between plants within the plots was 0.3 m and staggered in a zig-zag pattern (Fig. 1). Conventional fertilization regimes were used for each crop following the published agricultural extension guidelines and the recommendations of field station agronomists (Hernandez and Beaver 2015). A total of 1.81 kg of 10-10-10 +3Mg 1% trace elements were applied to all plots prior to planting. We used drip irrigation with 30.5 cm of hose between each emitter, which was positioned beneath the plastic mulch. Irrigation was done to saturation once or twice daily for up to 1 hour.

Treatment design

Each plot was randomly assigned to one of three treatments, Neem oil (chemical deterrent), mesh fence (physical barrier) or control (open field cultivation with no Neem or fence) using the package agricolae v 1.3-3 (De Mendiburu 2014) in R (R Core Team 2021). To assess the design and sample size needed to evaluate the impact of the green iguana on crop yield under these three treatments at two field sites, we performed a power analysis for a linear mixed model (Green and MacLeod 2016) in R. We first simulated pilot data for the Juana Diaz and Gurabo field sites. In each site, we simulated 10 plots for each treatment, each with 15 plants (i.e., to-taling 900 simulated lettuce plants). We then simulated that the highest probability of survival and growth would occur in our fenced treatments, with 1% death in the fenced treatment, 6% death for the neem treatment, and 8% death for the control treatment. If plants survived, we simulated the number of leaves for each plant and recorded the power to detect a significant fixed effect for different effect sizes using the R package simr v 1.0.7. From this analysis, we observed high power (>80%) to





Juana Diaz experimental site

Row arrangement and plant placement



Figure 1. A location of experimental sites in Puerto Rico and B view of study plots at the Juana Diaz Agricultural Experimental Station with a schematic showing the dimensions and spacing of plots and plants used in the study.

detect a significant difference for sample sizes of more than 10 plants per plot for a moderate effect size of 0.3 or larger between control and neem versus the fence treatment. R script for our power analysis is provided in the Suppl. material 2.

Based on the results of our power analysis, at both the Juana Diaz and Gurabo Experimental Stations, each treatment was replicated 10 times for a total of 30 plots of each crop, that is, 60 plots total for the two crops (i.e., 10 plots each of lettuce and cucumber in each of control, fence, and Neem treatments, Fig. 2). This resulted in a total of 450 plants of both lettuce and cucumber at each AES. All treatments had the same irrigation and fertilization regimes and, if necessary, pesticide applications. Cucumber plants were sprayed once with DiPel DF (a biological insecticide based on *Bacillus thuringiensis*) in Juana Diaz to treat a common foliar infection by Diaphnia spp. Plots were separated by 5.2 meters and placed parallel to the forested edge of the farm at each AES.

Christina N. De Jesús Villanueva et al.: Invasive Iguana iguana impacts on agricultural yield in Puerto Rico



Figure 2. Spatial arrangement of plots at the Juana Diaz Agricultural Experimental Station experimental site. Yield (Kg) and mortality (%) are shown for each plot. The border line pattern indicates the experimental treatment, red lines indicate the presence of green iguanas in the plot, and the rectangle fill color indicates the species of crop planted. Row 1 was the closest to the forest edge.

All treatments were subjected to the irrigation, fertilization, and pesticide application as described above and no further actions were taken for control plots. For the two experimental treatments, additional steps to deter green iguana herbivory were taken. In our first experimental treatment, we used a commercially available chemical deterrent called TrilogyTM (Certis USA), which is an organic foliar pesticide derived from Neem plants (*Azadirachta indica*). It was sprayed directly onto plants once per week by the station staff using a backpack sprayer for the duration of the experiment following the manufacturer's suggested dilution of 1%. For our second experimental treatment, we physically fenced in crops with a nylon monofilament fishing net (Lee Fisher Company, www.leefisherfishing.com). Net openings when fully tensed were 7.0 cm in size, the height of the net when tensed was 1.7 m, though in our treatment 0.6 m of the net was buried to prevent green iguanas from digging underneath the fence. To reduce the ability of green iguanas to climb on the surface, the nets were not fully taut and left hanging slightly off their posts (Fig. 1).

Data collection

For the duration of the experiment, we monitored green iguana presence and documented instances of herbivory through researcher observations and six camera traps (Foxelli Mod No. 57047, interspersed among plant treatments at edges and center of the experiment) at each experimental site. For cucumbers, we attributed plant herbivory to green iguanas when entire leaves were removed and only the petiole remained. If we observed leaves with other forms of damage (Suppl. material 1: fig. S1), this was not attributed to the green iguana. We measured the distance from the center of each plot to the forest adjacent to our experimental site as a measure of the minimum distance green iguanas would need to travel to reach the crops. To monitor plant growth during the experiment, we individually marked plants and counted the number of leaves on each plant daily during the first week of the experiment, then every other day for the remaining 31 days. If the number of leaves decreased over time, we considered this a sign of green iguana herbivory. Once the number of leaves surpassed 20 on a cucumber plant and the plants became entangled, and over 25 leaves on a lettuce plant, we monitored survival until harvest. After 42 days, we (4 persons) harvested the lettuce plants and cucumbers produced in each treatment. We documented the number of heads of lettuce produced in plots and their total weight, as well as the number of cucumbers and their total weight. Cucumbers had an additional second harvest based on the maturity of the fruit that occurred 10 days after the first harvest at Juana Diaz Experimental Station and two days after the first harvest at Gurabo Experimental Station.

Statistical analyses

We calculated the mean and standard deviation of plant growth and harvest yield for cucumber and lettuce plants using R in R studio (R Core Team 2021; RStudio Team 2021). We fit a Cox proportional hazards model using the *coxph* function in the R package survival (v3.2-7; Therneau, 2020) to test the effect of our treatments on plant survival over time. We fit a generalized linear mixed model (GLMM) by REML using the *lmer* function in the R package *lme4* (Bates et al. 2015). We included the variables treatment (i.e., control, Neem, and fence) and distance to the edge of the forest as fixed effects in our model and plant ID and plot within the field site as random effects. To evaluate the relationship between these variables and our response variables of plant growth and yield, we used the number of leaves as a measure of growth and either plant or fruit weight as a measure of yield. In the plant growth GLMM we included both plant ID and plot location as random effects, while for yield we only included plot location as a random effect. The latter variable, yield, was square-root transformed to improve normality based on our evaluation of its distribution using the function *descdist* from the R package fitdistrplus (Delignette-Muller and Dutang 2015). To compute confidence in the GLMM, we used the R package parameters, which allows the user to report standard error and p-values among the results of their statistical models (Lüdecke et al. 2020). We used the function *parameter*, which provides coefficients, standard errors, confidence intervals, t-values, and p-values at the intercept for fixed effects.

Results

During our pre-planting visual encounter survey (VES), we observed higher green iguana presence in Juana Diaz (289 lizards in 6 days, Fig. 1) compared to Gurabo (11 lizards in 5 days). The presence of green iguanas at the Juana Diaz Experimental Station was first noted on 8 September 2019, five days post-planting. This was confirmed by iguana tracks, camera trap images, and herbivory damage (Fig. 3).



Figure 3. Evidence of green iguana presence at the Juana Diaz Agricultural Experimental Station study site. Six camera traps were used to monitor for green iguana activity \mathbf{A} photo showing two green iguanas in a cucumber control plot \mathbf{B} photo documenting evidence of green iguana herbivory on a cucumber plant in a control plot. We considered plants with only a leaf petiole (and no leaf) as evidence of green iguana herbivory as opposed to instances of leaf damage indicative of insect herbivory \mathbf{C} photo showing claw and tail marks on the plastic mulch liner used to control weed growth.

By harvest, 22 out of 60 plots in Juana Diaz showed green iguana activity (Fig. 2). No green iguana evidence was found in Gurabo study plots during the experiment, though they were observed on the property before and after the study. Gurabo's mean yield data serves as an example of yields without iguana herbivory, showing no significant differences among treatments for lettuce or cucumbers (Suppl. material 1: fig. S2). We therefore focus the rest of our results on the Juana Diaz Experimental Station.

Photos from camera traps confirmed green iguana herbivory at Juana Diaz, with no other large herbivores observed (Suppl. material 1: fig. S3). One camera trap video captured a green iguana eating a lettuce leaf fragment in a control plot (Plot J1, Fig. 2), and the plant survived until harvest. In cucumber plots, iguana herbivory occurred in 17 of 30 plots, including 8 control, 7 Neem-treated, and 4 fenced plots.

Because we only documented or observed one incidence of herbivory on lettuce, and because there was no difference in growth or yield of lettuce as a function of our treatments (Fig. 2), we focus our results on cucumber plant growth and yield. At the Juana Diaz Experimental Station, cucumber plant growth based on the number of leaves was on average 18% higher in the fence treatment compared to the Neem treatment and 20% higher compared to the control plots (Table 1, Fig. 4). Cucumber plant mortality was 49 out of 450 plants or 11% (Fig. 2), with the lowest mortality occurring in fenced treatment plots (7 plants or 2%), followed by control plots (17 plants or 4%) and highest in the Neem treatment plots (25 plants or 6%). Results from the survival analysis (Fig. 4) showed that individuals in the fenced treatment had the lowest likelihood of mortality across most of the experiment (P = 0.046).

Our square-root transformed cucumber growth and yield data provided a better GLMM fit based on skewness (growth = 0.78, yield = -0.29), lower AIC values (Suppl. material 1: table S1) and in the Cullen and Frey graph of skewness versus kurtosis, which demonstrated approximation to normality. The GLMM analysis for cucumber growth showed that plants in the fenced treatments had higher growth than in the control plots (P < 0.001), but that plant growth in the Neem treatment did not differ from the control (P = 0.75). The GLMM analysis also showed that yield was higher for plants in the fenced plots (P < 0.001) compared to the control plots, but only nearly so in the Neem treatment compared to control (P = 0.071), and that plots farther from the forest edge had higher growth (P < 0.001) and yield (P = 0.052) (Table 2). Mean cucumber yield in the fenced treatment was 15.50 kg (34.17 lbs.; 551 cucumbers), which was over three times the yield of control plots (5.09 kg or 11.22 lbs.; 196 cucumbers) and twice the yield of Neem plots (7.63 kg or 16.82 lbs.; 337 cucumbers) (Table 3).

Table 1. Observations of green iguana occurrence made during visual encounter surveys (VES) along a 200-m transect adjacent to the fence line next to planting sites within the two Agricultural Experimental Station farms in Puerto Rico. Blanks are left for days where VES were completed in one site but not the other.

	2019 Visual Encounter Survey dates										
Farm	June 26	July 09	July 16	July 18	July 23	Aug 01	Aug 02	Aug 06	Aug 08	Aug 16	Total
Juana Diaz		43	51		46	55		52		42	289
Gurabo	0			6			0		2	3	11
										Total	300



Figure 4. At the Juana Diaz Agricultural Station **A** mean (\pm SD) for the number of leaves as a measure of cucumber growth by treatment. An increase in leaf number indicates plant growth **B** survival analysis comparing cucumber survival among treatments. Cucumbers in the fence treatment had a higher likelihood of survival over the course of the experiment (P = 0.046) **C** mean (\pm SD) for cucumber yield by treatment.

	Effect Estimate	SE	t value	Confidence interval lower 95	Confidence interval upper 95	р
Growth						
Intercept (Control)	1.82	0.08	21.76	[1.66]	[1.99]	<0.001
Cucumber Fence	0.20	0.05	3.82	[0.10]	[0.30]	<0.001
Cucumber Neem	0.02	0.05	0.32	[-0.08]	[0.12]	0.75
Distance to forest	0.0061	0.0015	3.99	[0.00]	[0.01]	<0.001
Yield						
Intercept	0.004	0.89	0.004	[-1.74	1.75]	0.997
Cucumber Fence	2.06	0.45	4.59	[1.18	2.94]	<0.001
Cucumber Neem	0.81	0.45	1.80	[-0.07	1.69]	0.071
Distance to forest	0.03	0.02	1.95	[0.00	0.06]	0.052

Table 2. Parameter estimates of fixed effects from generalized linear mixed model (GLMM) of cucumber growth and yield. We used the *lmer* function and fit the model by REML in the R package *lme4* (Bates et al. 2015).

Table 3. Mean (\pm SD), median (minimum, maximum) and total of cucumber yield (i.e., weightand number of cucumbers) for each treatment at the Juana Diaz Agricultural Experimental Station.

		Treatment		
Juana Diaz	Control (N = 20)	Fence (N = 20)	Neem (N = 20)	Total (N = 60)
Cucumber weight (Kg)				
Mean (SD)	5.09 (± 7.28)	15.50 (± 10.20)	7.63 (± 7.14)	9.41 (± 9.31)
Median [Min, Max]	0.670 [0, 21.5]	12.0 [3.00, 35.00]	8.00 [0, 24.50]	8.00 [0, 35.00]
Total yield	102	310	153	564
Number of cucumbers				
Mean (SD)	9.80 (± 14.10)	27.60 (± 15.00)	16.90 (± 12.90)	18.10 (± 15.60)
Median [Min, Max]	1.50 [0, 49.00]	25.00 [7.00, 52.00]	14.50 [0, 48.00]	15.00 [0, 52.0]
Total yield	196	551	337	1080

Discussion

Global agricultural production and food security is under immense pressure due to species invasions (Paini et al. 2016). In this study, we examined the impact that green iguanas have on cucumber and lettuce crop yield in Puerto Rico and tested techniques to mitigate the impact of this invasive reptile. Here we provide evidence of reduced crop yield and argue that the green iguana has the potential to significantly reduce agricultural yield in other important food crops in the tropics.

At a small research site (0.25 acres), we confirmed the detrimental effects of green iguanas on crop production previously reported in interviews with farmers (De Jesús Villanueva et al. 2022). Small scale farm data is considered a valuable source of information for understanding the effect of growing conditions on crop yield at larger scales (Huffman et al. 2015; Fry et al. 2017). The impact of the green iguana may have been conservative due to the regular presence of researchers, though it still led to significant differences in yield. In our study, the use of mesh fencing to exclude green iguanas increased cucumber yield by 50% when compared to using Neem and 67% compared to control plots. Abrams et al. (1976) estimated the yield of cucumbers (Gemini Variety) was 10 tons (9071.85 Kg) per acre at the Juana Diaz Experimental Station (then called Fortuna) before green

iguanas were present. In our experiment, we used ¼ of an acre, half of which was used for cucumber and further divided into the three treatments. Based on the area used in our experiment and Abrams et al.'s (1976) estimate, we expected a cucumber yield of 377.99 Kg. Our fenced treatment plots produced a yield of 310 Kg of cucumber, which is similar to those estimated by Abrams et al. (1976) 45 years ago without green iguana herbivory, whereas our yields for Neem (153 Kg) and control (102 Kg) plots were substantially less than the expected yield. Moreover, in the absence of green iguana herbivory, our Gurabo Experimental Station site showed no difference in cucumber yield among treatments (Fence = 179.60 Kg, Neem = 176.02 Kg, Control = 210.05 Kg, Suppl. material 1: fig. S2). This suggests that when green iguana herbivory does occur it has the potential to be a significant source of crop loss and ultimately economic loss.

The reductions in yield we observed in our Neem and control plots translate into potentially heavy economic losses for farmers who may be facing crop loss due to the green iguana and to the economy in general. By visiting five local food markets and distributors to determine the price of cucumbers, we were able to calculate the loss in revenue a Puerto Rican farmer would have faced on the island at the time of our harvest. In December 2019, a 25lb (11.33 Kg) box of cucumbers was being purchased wholesale at between \$19 and \$22 USD. Based on our harvest results, if a farmer would have used open field cultivation for cucumbers on one acre with plants experiencing green iguana herbivory, they would have sold their harvest at around \$4,429 USD (\$20.5 USD/ 11.33 Kg). In contrast, a farmer using mesh fencing to reduce green iguana herbivory would have sold their cucumbers for \$13,462 USD. Taking into account the cost of materials (estimated at \$134.00 for fencing) and labor (\$60 for 8 h in 2019), a net \$6,818 USD per harvest acre reduction in income could be used to argue in favor of implementing this management technique (Table 4). Estimated costs of implementing mesh fencing as a mitigation measure for a 1-acre plot are provided in the Suppl. materials. Though we provide the estimated costs of implementing this measure, farmers should consider netting that is set deeper into the ground and taller over the crops to fully exclude this lizard.

Our results suggest that Neem is ineffective at deterring green iguana herbivory and may lead to decreases in plant growth. We do not recommend the use of Neem as a mitigation technique for green iguana herbivory, as it does not lead to higher yields compared to those observed when no mitigation technique was used (i.e., control plots). The use of mesh fencing to protect crops from green iguana herbivory is effective at improving crop yield, although the effectiveness of this mitigation tactic may decrease over time. We observed green iguanas inside our fenced plots on multiple occasions. The reptiles were able to climb the

Table 4. Estimated crop (cucumber) revenue under different techniques to mitigate crop loss due to green iguana herbivory.

Cucumber revenue (USD)/acre ¹		
4,429.30		
13,461.80		
6,643.95		

¹Value per acre was estimated based on the yield produced at our Juana Diaz Agricultural Experimental Station, and the median value of cucumbers (20.5USD/ 11.33Kg) in Puerto Rico in December 2019.

fences, with some fenced treatments having more than one green iguana inside it at the same time (https://youtu.be/D7rIb71XF8Y). Weekly, or perhaps daily, maintenance of the fences is necessary throughout cultivation to ensure their integrity. To maintain fences in our experiment, we had to contend with weeds that would grow on our fence and pull it toward the ground, and heavy rains that washed soil away from the portion of mesh that was buried, resulting in the need to re-fit and seal gaps in the fences. At a farm, this would entail labor and material expenses that would need to be considered when scaling up to larger production. Durable fence material can be reused with careful planning to prevent knotting, which would reduce production costs. At larger scales, durability should be prioritized to ensure the investment in materials does not negate the revenue produced from seeking to increase yield. Methods to protect crops could be combined with other management techniques to further prevent crop loss such as done with other species (Rivadeneira et al. 2018). In birds, for example, exclusion netting is sometimes combined with lethal, auditory, and visual deterrents to prevent crop damage. Additional methods could be tested and used in combination with netting to attempt to increase crop protection.

Farmers should be provided with technical and financial assistance to implement green iguana mitigation strategies. This support might be particularly urgent for smaller farms that may suffer greater relative impacts. As documented here, the crop's plants grown in plots on the edge of our site closer to the forest (Fig. 2) were more susceptible to herbivory from green iguanas than those in the center of the field. Choosing to plant crops that are not part of the green iguana diet in edge plots may help decrease crop loss (De Jesús Villanueva et al. 2022), but this technique needs to be evaluated experimentally. In addition to recommendations on crop choice and cultivation location, considerations based on green iguana phenology should be made. As seen with other pest species (Murray 2008; Crimmins et al. 2020), phenology, or the relationship between a species yearly life cycle and the environment, can significantly impact the effectiveness of mitigation measures. Considering green iguana phenology (i.e., diet, reproductive cycle, relationship to daily and yearly environmental temperatures) when designing management recommendations may be critical to successful implementation. For example, farmers could be advised to avoid accumulating soil or plowing when green iguanas are in their nesting season to prevent the creation of nesting sites on the farm. Future work should focus on exploring the relationship between green iguana phenology and crop loss to ensure relevant management recommendations are made.

Our study focused on testing farm-level mitigation measures to decrease green iguana related crop loss. It is widely recommended, however, that preventing invasive species introduction altogether is a much more cost effective strategy than post-introduction management (Lodge et al. 2006). As a crop protection measure, we urge governments to strengthen their biosafety protocols to prevent green iguanas from becoming introduced. In regions where the risk of introduction has been identified (see Falcón et al. 2012, 2013), green iguanas should be included in invasive species monitoring programs. If programs have not been established, public species inventory programs (e.g., www.iNatularlist.org) can be used as a means of early detection (van den Burg et al. 2020). In places where a species has already been introduced, the potential for economic loss through herbivory should be addressed. If there is a lack of empirical evidence to support considering the green iguana as an agricultural pest, anecdotal accounts of the species damaging food crops should be considered as an early warning sign (Shah et al. 2020). Governmental and non-governmental agricultural management offices should keep close documentation of these anecdotal accounts to gain an understanding of the extent to which green iguanas could present a problem.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: JJK, CNDJV. Data curation: GPMP, CNDJV. Formal analysis: SVB, CNDJV, JJK. Funding acquisition: JJK, CNDJV. Investigation: GPMP, CNDJV, JJK. Methodology: CNDJV, SVB, JJK. Project administration: JJK, CNDJV. Resources: JJK, WG. Supervision: JJK. Visualization: SVB, CNDJV. Writing - original draft: JJK, CNDJV. Writing - review and editing: GPMP, WG, JJK, SVB, CNDJV.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary information

Authors: Christina N. De Jesús Villanueva, Gabriela P. Massanet Prado, Steven M. Van Belleghem, William Gould, Jason J. Kolbe

Data type: docx

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Link: https://doi.org/10.3897/neobiota.96.114925.suppl1

Supplementary material 2

A revision power analysis script R

Authors: Christina N. De Jesús Villanueva, Gabriela P. Massanet Prado, Steven M. Van Belleghem, William Gould, Jason J. Kolbe

Data type: R file

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Supplementary material 3

Revision statistical analysis script R

Authors: Christina N. De Jesús Villanueva, Gabriela P. Massanet Prado, Steven M. Van Belleghem, William Gould, Jason J. Kolbe

Data type: R file

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Link: https://doi.org/10.3897/neobiota.96.114925.suppl3

Supplementary material 4

Revision study data

Authors: Christina N. De Jesús Villanueva, Gabriela P. Massanet Prado, Steven M. Van Belleghem, William Gould, Jason J. Kolbe

Data type: csv

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Research Article

Winter leaf phenology differences facilitate selective control of an invasive plant species by herbicide

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Abstract

Herbicide control of invasive plant species is generally efficient. However, there is a likelihood of the excessive application of non-selective herbicides that kill co-occurring native species and cause environmental toxicity. We present a case study on the control of the invasive exotic Solidago canadensis with photosynthetically active leaves in winter by applying glyphosate. This approach improves herbicide control efficiency, while preventing harm to most co-occurring native plants. We quantified the winter leaf phenology and photosynthetic capacity of S. canadensis and two commonly co-occurring native species. We tested the effects of glyphosate and competition on S. canadensis and native Imperata cylindrica with contrasting winter leaf phenology in both pot and field experiments. Finally, we surveyed the life forms and winter leaf phenology of most co-occurring plant species in eastern China to determine whether most co-occurring species and S. canadensis differ in winter leaf phenology. Old leaves withered much later and new leaves developed much earlier in S. canadensis than in the two co-occurring species. Both the old and new leaves of S. canadensis had high photosynthetic capacity in winter. In summer, glyphosate suppressed the growth of S. canadensis by 20.1-59.5% and growth of I. cylindrica to a greater extent (by 57.6–91.7%), whereas winter application of glyphosate at a certain concentration suppressed the growth of S. canadensis by 91.4-95.6% (the efficiency was higher than summer application), but had no impact on I. cylindrica. Glyphosate application in winter alleviated competition stress from S. canadensis on I. cylindrica. We conclude that winter glyphosate application can increase the selectivity and efficiency of chemical control of invasive S. canadensis, which may shift the competition balance towards native species and favour native vegetation recovery in sites invaded by S. canadensis. The principles of this approach can be applied to any scenario where invasive species and co-occurring species have distinct phenological niche separation.

Key words: Clonal plants, competition, glyphosate, photosynthesis capacity, rhizome, *Solidago canadensis*

Introduction

Biological invasions are amongst the greatest threats to indigenous biodiversity, ecosystem services and economic development globally (van Kleunen et al. 2015; Early et al. 2016; Head 2017; Adomako et al. 2019; Roiloa et al. 2020). Invasive exotic plant species are typically larger, accumulate more biomass, form



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considerable monocultural patches and, eventually, exclude native plants from invaded sites (van Kleunen et al. 2010; Drenovsky et al. 2012; Godoy et al. 2012; Wang et al. 2017, 2019). Dramatic changes in local vegetation structure caused by the invasion of exotic plant species can lead to fundamental changes in ecosystem functions, which are usually degraded by biodiversity loss (Hejda et al. 2009; Vilà et al. 2011; Peller and Altermatt 2024).

Management of invasive species — by inhibiting growth, population increase and expansion — is necessary for biodiversity conservation (Mačić et al. 2018; Gentili et al. 2021; Sherrill et al. 2022). However, tackling exotic invasive species is challenging because they usually have a high growth capacity and resistance to stresses and disturbances (van Kleunen et al. 2010; Drenovsky et al. 2012; Godoy et al. 2012). Common practices for controlling invasive species include physical removal by pulling out or mowing and chemical control by spraying herbicides (Sebastian et al. 2017; Mahmood et al. 2018), biological control by introducing natural enemies (Pratt et al. 2013) and restoration of native species to increase ecological resistance (Byun et al. 2018). These approaches vary in their cost and effectiveness for the eradication of exotic invasive plants, depending on the species in question and the stage of socioeconomic development in the area (Weidlich et al. 2020).

Phenology is an essential functional trait that can be used to distinguish invasive species from native species, based on their different temporal niches throughout the season (Fridley 2012; Wolkovich and Cleland 2014; Park et al. 2024). Several studies have reported that exotic species have extended growth season length, due to an earlier start of growth period and/or later senescence (Polgar et al. 2013; Smith 2013; Wilsey et al. 2018). These differences have been considered an important factor driving invasion success (Fridley 2012; Rejmánek 2013) by filling vacant temporal niches, enhancing resource pre-emption or growth (priority effects or prolonged growth period) (Wolkovich and Cleland 2011) or, possibly, via alteration of allelopathy, apparent competition or pollination (Smith 2013; Smith and Hall 2016). The impact of phenological differences on exotic species invasion can be more pronounced with global changes such as increased nitrogen deposition (Valliere et al. 2022). Furthermore, competition may also alter phenology, for example, a reduction in lifespan (Levine et al. 2024). Thus, phenological niche differences may offer resource managers the opportunity to control exotic species, while protecting native species (Wolkovich and Cleland 2011; Hess et al. 2019; Taylor et al. 2020).

Amongst these different approaches, the application of herbicides is common and usually effective (Wagner et al. 2017; Mahmood et al. 2018). However, most herbicides do not have high specificity, resulting in a "non-target effect", in which native plant species may be damaged as much as or greater than the target species (Gibson et al. 2019; Peterson et al. 2020). Furthermore, excessive herbicide use may increase the risk of environmental toxicity (Richmond 2018; Peterson et al. 2020; Gandhi et al. 2021) and promote the development of herbicide-resistant populations of exotic species (Baucom 2019). Gu et al. (2017) reported that application of glyphosate at high doses in the growing season could depress growth and caused mortality of a common co-occurring plant species to a greater extent than that of invasive *Solidago canadensis*. Consequently, when applying chemical controls, both the dosage of herbicides and their potential harm to native species must be reduced as much as possible. Progress has been made in the use of phenological differences to control invasive plant species. Wainwright et al. (2012) showed that manipulation of earlier rainfall stimulated early germination of exotic species in unfavourable seasons, but not of native species, which led to high mortality of exotic species and may favour native species restoration. Planting early native species early in the season or fast-growing species after the removal of invasive species can effectively suppress the growth of exotic species (Smith et al. 2021; Kumar et al. 2024). It has been suggested that phenological differences can be utilised to increase the selectivity of herbicides in controlling invasive species during seasons when invasive species are active and native species are inactive (Wolkovich and Cleland 2011). Caplan et al. (2018) showed that herbicide application can still be effective in late autumn for control of invasive *Lonicera* species with delayed leaf senescence. It remains unclear how herbicide control of invaders may affect neighbouring native species and their interactions with invaders.

Solidago canadensis (Asteraceae), commonly known as Goldenrod, is native to North America, but has become invasive in Asia, Europe and Australia (Perera et al. 2021; Qiang et al. 2021). Due to its highly competitive capacity, S. canadensis replaces native plant species, forming large patches of monocultures and causing a decrease in the diversity of native plant species (Szymura and Szymura 2016; Adomako et al. 2022; Ye et al. 2022; Xie et al. 2023). To date, no efficient or environment-friendly methods have been developed to control this species. In eastern China, invasive S. canadensis has a winter phenology that differs from that of the co-occurring species. Mature leaves of S. canadensis senesce much later in the season than the leaves of most co-occurring plant species and new ramets and leaves appear in early autumn — far earlier than in most native species, which do not start growing until early spring. A preliminary test also showed that both old but viable leaves and newly-grown leaves of S. canadensis are photosynthetically active in winter, whereas the completely senescent leaves of most native co-occurring plant species are inactive. As most herbicides are only effective after being absorbed by green leaves or viable roots (Sterling 1994; Nandula and Vencill 2015), it is possible that herbicides may only injure S. canadensis, leaving native plant species with dormant aboveground parts — unaffected. Although it is recognised in practice that some herbicides, such as glyphosate, can still be effective in winter as long as the air temperature is above a certain threshold value, there are very few studies on the effects of herbicide application on target plants in winter. Badalamenti et al. (2015) reported significant seasonal effects of herbicides on the mortality rate and re-sprouting ability of invasive Ailanthus altissima under Mediterranean climatic conditions, indicating the possibility of improving control efficiency by selecting optimal seasons. Furthermore, studies on the effects of herbicides on growth and competition between invasive plants and co-occurring native plants with contrasting winter phenologies have not yet been reported.

In the present study, we tested the feasibility of applying glyphosate, a commonly used herbicide (Duke and Powles 2008; Richmond 2018; Gandhi et al. 2021), to control *S. canadensis*, while avoiding harming co-occurring species in winter. First, we quantified the differences in winter leaf phenology and photosynthetic capacity between invasive *S. canadensis* and two co-occurring species. Second, in both field and pot experiments, we compared the responses of *S. canadensis* and a common native co-occurring species with contrasting winter leaf phenology to glyphosate application during summer and winter. We hypothesised that winter glyphosate application would inhibit the growth of *S. canadensis* with photosynthetically active leaves, but not that of the native species with dead leaves. Finally, we surveyed the life forms and related winter leaf phenology of most co-occurring species of *S. canadensis* in eastern China to clarify whether this strategy can be applied to *S. canadensis* at various sites.

Materials and methods

Study sites

The pot and field experiments described below were conducted in Fuyang, Hangzhou, Zhejiang Province, China (29.7–30.2°N, 119.4–120.1°E), where the invasive plant *S. canadensis* is widely distributed. This area is characterised by a typical subtropical monsoon climate with four distinct seasons. The terms summer and winter in this study are defined according to the Chinese National Standards (GB/T 42074-2022) (Standardization Administration of China 2022). Specifically, summer and winter start when daily mean temperature of five consecutive days is above 22 °C and below 10 °C, respectively. Summer (May – September) is hot and wet and winter (December – February) is relatively cold and dry. During winter, the mean daily maximum air temperature is 11 °C in December, 8 °C in January and 11 °C in February (Suppl. material 1). The mean daily minimum air temperatures in these three months are 3, 1 and 3 °C, respectively and the mean monthly precipitation is 51, 72 and 85 mm, respectively (Suppl. material 1). The mean daily air temperature at the study site during the study period is shown in Suppl. material 2.

Autumn-winter leaf phenology of S. canadensis and two co-occurring natives

The leaf phenology of *S. canadensis, Artemisia lavandulaefolia* (Asteraceae) and *Imperata cylindrica* (Poaceae) was quantified from October 2022 to February 2023. *A. lavandulaefolia* and *I. cylindrica* are common native species co-occurring with *S. canadensis* in eastern China (Ye et al. 2019). All three species are perennial, can reproduce both sexually and asexually (clonally) by producing rhizomes and may compete intensively in the field.

In March 2022, *S. canadensis, A. lavandulaefolia* and *I. cylindrica* seeds were germinated in pots ($5 \times 5 \times 6$ cm) filled with a mixture of vermiculite, sand and peat (2:1:1 in volume) in a growth chamber at 24/18 °C and photosynthetic active radiation of 250 µmol·m⁻²·s⁻¹. On 8 March 2022, after two weeks, seedlings were transplanted to plots (1×1 m) in an open site in the experimental garden of the Institute Subtropical Forestry, Chinese Academy of Forestry in Fuyang. The open site was created by pulling out all the plants. The seedlings were planted in the centre of the plots and each plot was planted with only one seedling from one of the three species. Ten plots were established for each species (n = 10). The distribution of the three species and replicates followed a completely random design as the plots were located within a small distribution range with relatively low environmental heterogeneity. The plants was a type of yellow-red ferrosol (pH = 6.5 and the availability of N, P and K is 69.14 mg/kg, 0.84 mg/kg and 153.0 mg/kg,

respectively). During the growth period, the non-target plants in the plots were weeded out to prevent possible interference on the target plants.

From 5 October 2022, the beginning of autumn (mean temperature between 10 and 22 °C for five consecutive days), the number of viable old leaves (leaves on the ramets produced before the autumn) were recorded for each plant every two days. An old leaf with an estimated more than 50% of the total leaf area maintaining green colour was considered viable. The duration from the start of autumn to the day of complete senescence of the old leaves was calculated for each individual plant. On 30 November 2022, at the beginning of winter (mean temperature below 10 °C for five consecutive days), the number of new ramets (produced from the start of autumn), new leaves (appearing on the new ramets) and old viable leaves were counted for each plant. It should be noted that the leaf phenology of *S. canadensis* may vary with region, as different regions may differ greatly in their local climate. This variation in the timing of old leaf senescence and new ramet and leaf production in autumn and winter may influence the chemical control efficiency described below.

Winter photosynthetic characteristics of *S. canadensis* and *A. lavandulaefolia*

As the aboveground part of *I. cylindrica* was completely senescent in winter, photosynthetic capacities were characterised for *S. canadensis* and *A. lavandulaefolia* using the same individuals as described in the previous section. Photosynthesis was measured from 10 December to 10 February 2022, using a Li-6800 device (LI-COR, Inc., Lincoln, Nebraska). Light response curves of photosynthetic rate were measured for one old leaf and one new leaf for each of the six randomly selected plants of each species (n = 6). The selected old leaves were located approximately at the middle position of the old shoots and new leaves were from the rosettes of newly-produced ramets. The photosynthetic rate (A_{sat}) at the light saturation point (LSP), light compensation point (LCP), apparent quantum yield (AQY) and dark respiration rate (R_d) were calculated, based on the light response curves (Ye 2007).

Effects of glyphosate and competition on *S. canadensis* and *I. cylindrica*

The effects of glyphosate and competition on growth of *S. canadensis* and *I. cylindrica* were investigated in pot and field experiments. *S. canadensis* and *I. cylindrica* seeds were germinated and cultivated as described above. The seedlings were transplanted to pots $(18 \times 18 \times 19 \text{ cm})$ filled with a mixture of silt loam soil and peat (the availability of N, P and K is 112.2 mg/kg, 1.7 mg/kg and 190.0 mg/kg, respectively) in the experimental garden.

Additive design was used to test the competitive effects of *S. canadensis* and *I. cylindrica*. One seedling of *S. canadensis* and *I. cylindrica* was grown together (with interspecific competition) or alone (without interspecific competition from each other, control) (Fig. 1). Glyphosate was applied on 5 July 2022 (two months after the start of the summer in 2022) or on January 30 (two months after the start of winter in 2022) (Fig. 1). A 20 ml glyphosate solution was applied to each plant at three levels: 0 (distilled water as control), 0.9 and 1.8 ml/l, using mini-plastic spray bottles (maximum volume: 50 ml). Glyphosate, Monsanto Company, U.S.A)



Figure 1. Overview of the experiment. Illustrations of the competition treatments (competition between invasive *Solidago canadensis* and native *Imperata cylindrica*) and the glyphosate treatments in the pot experiment in summer and winter.

with distilled water to the aforementioned concentrations. For the mixed-species pots, *S. canadensis* and *I. cylindrica* plants were temporarily separated with a plastic plate to prevent over-spraying of glyphosate. Ten replicates were performed for each competition × glyphosate treatment during each season, with a total of 90 pots (three competition treatments × three glyphosate treatments × 10 replicates) for each season. The treatments and replicates were randomly distributed and the positions of the pots were regularly re-arranged to reduce the potential effects of environmental heterogeneity in the experimental garden. The plants, treated with glyphosate in July (summer), were harvested on 5–10 November 2022 and those treated in January (winter) were harvested on 5–10 June 2023. The plants were dried at 80 °C to a constant weight and weighed to obtain dry mass.

In the field experiment, S. canadensis and I. cylindrica seedlings were cultivated in 2021 and transplanted into field plots (1 × 1 m) on 24 March 2022. The site is located at an abandoned agricultural field with a kind of red-yellow ferrosol (pH = 6.5 and the availability of N, P and K was 152.6 mg/kg, 2.3 mg/kg and 55.9 mg/kg, respectively). Two weeks before transplantation, all the plants in the plots were removed using glyphosate. A substitution design was used for the competition treatments. In the monoculture treatment, four S. canadensis or I. cylindrica plants were grown in a plot (without interspecific competition from each other). In the mixture treatment, two plants, S. canadensis and I. cylindrica, were grown in a plot (with interspecific competition). Within each plot, the plants were arranged at intervals of 50 cm. On 15 January 2022, for each species, we sprayed 120 ml of glyphosate solution (0.9 ml/l) on six randomly selected plots and 120 ml distilled water (control without glyphosate) on the other six plots. A total of 36 plots were established (three competition treatments × two glyphosate treatments × six replicates). A prior test showed that the soil physicochemical properties were relatively homogeneous between the plots and a completely random design was used in the experiment. Non-target plants were removed by pulling them out during the growth period. Four months later, on 15-17 May, the ramet number and shoot height of three randomly selected ramets in each plot were measured and the cover of S. canadensis and I. cylindrica in each plot was visually estimated.
Life forms and winter leaf phenology of co-occurring native species in the wild

The diversity of native species co-occurring with S. canadensis was surveyed from autumn to winter in 2023 in Shanghai City, Zhejiang Province, Jiangsu Province and Anhui Province, where it is characterised by a typical subtropical monsoon climate and S. canadensis has the highest distribution. We randomly selected 120 sites from these four regions, based on their latitude and longitude from these four regions. S. canadensis plants typically grow along roadsides, riversides or in old fields. The possible habitats of S. canadensis were investigated within a distance of 1 km from the centre of each site. Stands of *S. canadensis* with a patch area of > 2 m^2 were found at 88 sites. A 1×1 m plot within each patch of *S. canadensis* and a $3 \times$ 3 m plot outside, but closely connected to, the S. canadensis patch were set up. The life forms and winter leaf phenology of all plant species in the plots were recorded by observation during the site survey, as well as the description of phenology and life form in Flora of China (Editorial Committee of Flora of China 1994). Winter leaf phenology was determined another two to three times at some sites near the location of the pot experiment and the field experiment throughout the winter, if the winter phenology of some plant species could not be determined in the first survey.

Statistical analyses

One-way ANOVA was used to test the effect of species (*S. canadensis, A. lavandulaefolia* and *I. cylindrica*) on autumn-winter leaf phenology (duration of complete leaf senescence and the number of old leaves, new leaves and new ramets at the start of winter). The differences in photosynthetic parameters (A_{sat}, LSP, LCP, AQY and Rd) between the old and new leaves of *S. canadensis* and *A. lavandulaefolia* were also analysed using one-way ANOVA. The effects of competition, glyphosate application and their interactions were analysed using two-way ANOVA for both the pot and the field experiments. Duncan's test was used for multiple comparisons, if the effect was significant. The biomass and percentage (e.g. cover) data were log-transformed or arc-sine-transformed if they did not conform to parametric assumptions. The data illustrated in the figures and tables were not transformed. All the analyses were performed using the IBM SPSS Statistics Version 27 (IBM Corp., Armonk, New York, USA).

Results

Autumn-winter leaf phenology differences between S. canadensis and natives

S. canadensis differed greatly from the two co-occurring native species, *A. lavan-dulaefolia* and *I. cylindrica*, in terms of autumn-winter phenology (Fig. 2). From autumn to winter, the old leaves died the earliest in *I. cylindrica* and the latest in *S. canadensis* (Fig. 2A). No new ramets or leaves were present in *I. cylindrica* at the start of the winter of 2022. In contrast, new ramets and leaves were produced in *S. canadensis* and *A. lavandulaefolia* and viable, old leaves were found in these two species. The numbers of new ramets, new leaves and viable old leaves were significantly greater in *S. canadensis* than in *A. lavandulaefolia* (Fig. 2B).



Figure 2. Winter leaf phenology of *Solidago canadensis* and the two native species (*Artemisia lavandulaefolia* and *Imperata cylindrica*) **A** time (days) to complete dieback of old leaves (the duration was calculated from the start of autumn **B** number of viable aboveground organs (new ramets, new leaves and old leaves) per plant at the start of winter. Mean \pm standard error are given. Different letters indicate significant differences (P < 0.05).

Photosynthetic characteristics of *S. canadensis* and *A. lavandulaefolia* in winter

Both *S. canadensis* and *A. lavandulaefolia* plants were able to efficiently photosynthesise during the winter (Table 1). However, the two species differed significantly in their photosynthetic characteristics (Table 1). For both species, the new leaves had a higher photosynthetic capacity than the old leaves (P < 0.05). The old leaves of *S. canadensis* had a much lower LSP than those of *A. lavandulaefolia* (P < 0.05), although the new leaves had similar LSP (P > 0.05). The new leaves of *S. canadensis* had significantly higher A_{sat} than those of *A. lavandulaefolia* (P < 0.05). LCP and R_d were higher in the new leaves of *S. canadensis* than in those of *A. lavandulaefolia* (P < 0.05).

Growth responses to competition and glyphosate in *S. canadensis* and *I. cylindrica*

The growth responses of *I. cylindrica* and *S. canadensis* to glyphosate and competition varied between the two pot experiments conducted in summer and winter (Table 2). In summer, the biomass of *I. cylindrica* was significantly affected by glyphosate, competition and their interaction, whereas the biomass of *S. canadensis* was only affected by glyphosate (Table 2). In summer, the biomass of *S. canadensis* decreased by 20.1% and 59.5% in the 0.9 ml/l and 1.8 ml/l treatments, respectively, compared with the control (Fig. 3A). For *I. cylindrica*, the biomass decreased by 57.6% and 91.7% in the 0.9 ml/l and 1.8 ml/l treatments, respectively, to a much greater extent than that for *S. canadensis* (Fig. 3B). Interspecific competition significantly decreased the biomass of *I. cylindrica* in the control and the 0.9 ml/l treatments in summer, but not that of *S. canadensis* in any of the glyphosate treatments (Fig. 3A, B). In contrast, in winter, the biomass of *I. cylindrica* was hardly negatively affected by glyphosate, but was significantly affected by competition

Table 1.	Photosynthetic	characteristics	of Solidago	canadensis	and Artemisia	lavandulaefolia	during
winter.							

¥7 · 11	S. can	adensis	A. lavandulaefolia			
Variable	New leaf	Old leaf	New leaf	Old leaf		
LSP	960.0 ± 97.9a	533.3 ± 42.1b	963.6 ± 52.6a	810.0 ± 79.5a		
A _{sat}	14.4 ± 0.7a	7.8 ± 0.6c	12.4 ± 0.3b	8.1 ± 0.4c		
AQY	0.064 ± 0.002a	0.055 ± 0.002ab	0.051 ± 0.002bc	0.042 ± 0.005c		
LCP	29.7 ± 4.1a	25.7 ± 2.2ab	20.0 ± 1.7b	18.2 ± 2.9b		
R _d	2.0 ± 0.3a	1.5 ± 0.1b	1.2 ± 0.1bc	0.9 ± 0.1c		

LSP (μ mol·m⁻²·s⁻¹), light saturation point; Asat (μ mol·m⁻²·s⁻¹), photosynthetic rate at LSP; AQY, apparent quantum efficiency; LCP (μ mol·m⁻²·s⁻¹), light compensation point; Rd (μ mol·m⁻²·s⁻¹), dark respiration rate. Values are presented as mean ± standard error. Different letters indicate significant differences (P < 0.05).

Table 2.	Effects	of glyphosate	application	and	competition	on	Solidago	canadensis	and	Imperata
cylindrica	biomas	s.								

6	EC .		S. canadens	is	I. cylindrica		
Season	Effect	DF	F	Р	DF	F	Р
Summer	Competition	1	1.2	0.276	1	227.7	< 0.001
	Glyphosate	2	19.8	< 0.001	2	112.5	< 0.001
	Interaction	2	0.28	0.759	2	104.0	< 0.001
Winter	Competition	1	2.2	0.142	1	8.9	0.004
	Glyphosate	2	247.2	< 0.001	2	2.7	0.077
	Interaction	2	0.2	0.814	2	10.0	< 0.001

and the interaction between glyphosate and competition, whereas the biomass of *S. canadensis* was only affected by glyphosate (Table 2). In winter, the 0.9 ml/l and 1.8 ml/l glyphosate decreased the biomass of *S. canadensis* by 91.4% and 95.6%, respectively (Fig. 3C), whereas the biomass of *I. cylindrica* was not decreased significantly by the glyphosate treatments (Fig. 3D). In winter, interspecific competition did not reduce the biomass of *S. canadensis* in any of the three glyphosate treatments; however, it did reduce the biomass of *I. cylindrica* in the control and 1. 8 ml/l treatments (Fig. 3C, D).

The field experiment also showed that *S. canadensis* and *I. cylindrica* differed in their responses to competition and glyphosate application during the winter (Table 3 and Fig. 4). Glyphosate application in winter reduced the shoot height, ramet density and cover of *S. canadensis*, measured in spring, by 19.9%, 48.9% and 32.1%, respectively, in the monoculture treatment and by 25.4%, 29.9% and 21.1%, respectively, in the mixture treatment (Table 3 and Fig. 4). However, glyphosate application during the winter did not significantly affect any of the spring growth measures of *I. cylindrica* (Table 3 and Fig. 4). The competition between *S. canadensis* and *I. cylindrica* negatively affected the ramet number and cover of both species. The strength of the effects of competition on ramet number seemed to be similar for the two species, but the effects on cover tended to be much stronger in *I. cylindrica* (Table 3). The growth parameters were not significantly affected by interactions between glyphosate application and competition, except for the ramet number of *S. canadensis*, which was significantly reduced by glyphosate in the monoculture treatment, but not in the mixture treatment.



Figure 3. Biomass of *Solidago canadensis* and *Imperata cylindrica* in response to glyphosate and competition in summer and winter **A**, **C** invasive *S. canadensis* **B**, **D** native *I. cylindrica*. Glyphosate was applied at three levels: CK (distilled water, without glyphosate), 0.9 and 1.8 ml/l, with a total of 20 ml glyphosate solution for each plant in either summer (**A**, **B**) or winter (**C**, **D**). In each pot, one *I. cylindrica* or *S. canadensis* plant was grown alone (without competition) or together (with competition). Each column represents the mean \pm standard error for each species. Different letters indicate significant differences (P < 0.05) for each treatment combination.

D	Effect		S. canadens	is	I. cylindrica			
Parameter		DF	F	Р	DF	F	Р	
Shoot height	Competition	1	6.191	0.02	1	1.9	0.180	
	Glyphosate	1	35.704	< 0.001	1	0.3	0.560	
	Interaction	1	0.254	0.619	1	2.3	0.148	
Ramet number	Competition	1	35.055	< 0.001	1	27.1	< 0.001	
	Glyphosate	1	16.518	< 0.001	1	3.2	0.087	
	Interaction	1	6.543	0.017	1	0.1	0.997	
Cover	Competition	1	29.9	< 0.001	1	70.5	< 0.001	
	Glyphosate	1	15.7	0.001	1	0.9	0.331	
	Interaction	1	3.0	0.096	1	0.7	0.409	

Table 3. Effects of glyphosate application in winter and competition on spring growth of *Solidago* canadensis and Imperata cylindrica in the field.



Figure 4. Spring growth of *Solidago canadensis* and *Imperata cylindrica* treated with glyphosate in winter and competition in the field. Invasive *S. canadensis* (**A–C**) or native *I. cylindrica* (**D–F**) were grown either in monoculture (without interspecific competition) or in a mixture (with interspecific competition between each other); a total of 120 ml distilled water (without glyphosate, -glyphosate) or 0.9 ml/l glyphosate solution (with glyphosate, +glyphosate) was applied to each plot. The values in the figure indicate mean ± standard error for each treatment combination. Different letters indicate significant differences (P < 0.05) for each growth parameter and NS indicates an insignificant difference between the treatments.

Diversity and life forms of natives co-occurring with *S. canadensis* in the wild

Native plant species that co-occurred with *S. canadensis* in the wild were divided into four groups according to their origin and life form during winter: native annuals with complete aboveground dieback, native perennials with complete leaf dieback, native perennials with viable leaves and invasive annuals with viable leaves. Only two co-occurring species, *Artemisia lavandulaefolia* and *Rumex japonicus*, had viable leaves during the winter (Table 4).

Life forms	Leaf vitality	Major co-occurring plant species
Native annuals	Dieback	Setaria viridis, Glycine soja, Lactuca indica, Solanum nigrum, Hemisteptia lyrata, Youngia japonica, Pterocypsela indica
Native perennials	Dieback	Phragmites australis, Imperata cylindrica Miscanthus sacchariflorus, Humulus scandens, Pueraria lobata
Native perennials	Vital	Artemisia lavandulaefolia, Rumex japonicus
Exotic annuals	Vital	Erigeron philadelphicus, Erigeron annuus Conyza canadensis

Table 4. Major co-occurring plant species of Solidago canadensis and their life forms.

Discussion

We showed that glyphosate effectively controlled photosynthetically active *S. canadensis*, a highly competitive invader, but did not affect dormant *I. cylindrica* in winter in a subtropical climate. These results support the hypothesis that glyphosate application can selectively and more efficiently depress *S. canadensis*, while avoiding harm to dormant native species, thus shifting the balance of competition towards the co-occurring native plants.

Winter phenology of S. canadensis and its co-occurring species

The old leaves of *S. canadensis* withered much later and produced new ramets and leaves much earlier than the two co-occurring species did. The results of the large-scale field survey also indicated that most of the co-occurring species of *S. canadensis* persisted throughout winter without viable leaves. The extension of growth period is consistent with many studies, showing that invasive species generally have longer growing seasons, which may endow them with the advantage of fixing more carbon and becoming more competitive (Wolkovich and Cleland 2011; Fridley 2012).

Temperature and/or apical dominance may control earlier vegetative growth of invasive S. canadensis from autumn to winter. This species begins new vegetative growth as early as early October in a subtropical climate, concurrent with the start of autumn and the flowering of S. canadensis (Cheng et al. 2021), when the mean maximum air temperature sharply decreases in the subtropical region (Suppl. material 1). S. canadensis originates from the temperate regions in North America with mild summers and the much higher air temperature in the subtropical region in eastern China may inhibit ramet production and flowering in S. canadensis (Cheng et al. 2021). The second explanation for the initiation of new ramet growth in autumn is that the transition from vegetative growth to flowering in S. canadensis releases belowground buds from apical dominance (Chung et al. 1994). The presence of apical dominance from apical buds or shoot tips inhibits the outgrowth of axillary buds located below (Cline 1997; Beveridge et al. 2023). S. canadensis shows a strong ability to regenerate from rhizome fragments, axillary buds and adventitious buds in stem bases or rhizomes if the aboveground part is clipped (Huang et al. 2007; Weber 2011), suggesting a strong dominance of apical buds over axillary buds and belowground parts. This apical dominance might disappear when vegetative shoots transform into inflorescences (Chung et al. 1994).

Interspecific variations in winter phenology may indicate different strategies to cope with unfavourable winter coldness in subtropical regions (Estiarte and Peñuelas 2015; Varpe 2017). Although winter is relatively mild in the region,

the extreme minimum temperature can be as low as -9 °C, as recorded in early 2016. The maintenance of green leaves in *S. canadensis* during unfavourable seasons against the risk of freeze injuries indicates its high freezing tolerance, possibly owing to its origin in temperate climates with lower air temperatures in winter, as well as polyploidisation (Lu et al. 2020; Cheng et al. 2021). In contrast, most co-occurring herbaceous plant species overwinter as dormant seeds or perennial tissues (mostly underground) under subtropical conditions, representing an escape strategy (Tang et al. 2016; Varpe 2017).

It should be noted that the phenology of S. canadensis and its neighbours and the consequent discrepancies may vary substantially with local climate and climate change (Park et al. 2024), either through plastic phenotypic responses or evolutionary adaptations. In contrast to the autumn-winter phenology observed in this study, S. canadensis does not produce new ramets from rhizomes until the next spring in its native range, such as in Ontario, if undisturbed (Werner et al. 1980), likely due to much shorter growing season and relatively lower winter temperatures. Peng et al. (2019) showed that warming could extend leaf lifespan and enhance growth of S. canadensis and an invasive population in China was more sensitive in these responses than a native population from the USA in a common garden experiment. Cheng et al. (2021) also showed that polyploidisation improves heat tolerance and delays flowering in the invaded ranges, which may sequentially delay dieback of old shoots during the global expansion of S. canadensis from its temperate-climate native ranges in North America to hot-summer climate in an introduced range. These studies suggest that both plastic phenotypic and genetic changes in the invasive species may occur in response to different climate-related environments. Furthermore, studies have shown that some exotic invaders are more plastic in their responses to climate change than natives, which may increase their competitive advantages (Hulme 2011; Polgar et al. 2013) and pose challenges for future invasion management.

Effects of glyphosate application on growth and competition

This study indicates much stronger competitive capacity of *S. canadensis*, compared to that of one of its common neighbours, *I. cylindrica*, consistent with its high performance in the field (Szymura and Szymura 2016). The growth of *S. canadensis* was never reduced by competition from *I. cylindrica*, regardless of the application of glyphosate in either summer or winter. In contrast, the growth of *I. cylindrica* was almost consistently reduced by competition from *S. canadensis* in summer and in the control treatment in winter. Diverse mechanisms, including the extended growth season observed in this study, may jointly contribute to high competitive ness (Cheng et al. 2021; Wang et al. 2023; Li et al. 2024). The high competitive ability of this invader also suggests that it is not feasible to resist *S. canadensis* invasion via intensified competition from native plant species alone, without imposing further strong disturbances on the invader and simultaneously with less impact on natives, such as herbicides (Munné-Bosch and Santos 2024).

Our study suggests that chemical control of *S. canadensis* may modify the competition balance if the invader and the native neighbours are equally treated, but respond differentially to herbicides in different seasons. In summer, glyphosate may exacerbate further invasions, because *S. canadensis* is less affected and may recover more quickly than native species. *S. canadensis* was more tolerant to

glyphosate than *I. cylindrica* in summer, which was consistent with the findings of Gu et al. (2017). Invasive plant species may be more tolerant to herbicides because of their larger plant size or higher regrowth capacity (Enloe and Netherland 2017). Other studies have indicated that invasive exotic species are normally more intrinsically stress tolerant or have higher capacities to recover from disturbances or stresses (Alpert et al. 2000; Godoy et al. 2011; Wang et al. 2017, 2019, 2024; Li et al. 2022). In summer, it is also likely that temporary release of empty niches by disturbances may facilitate establishment of more targeted and non-targeted exotic invasive species.

However, winter herbicide treatment may favour native species in the long term. The ranking of tolerance to glyphosate was reversed in winter for S. canadensis and its neighbours. Physiological activity may play an important role in the tolerance of species to herbicides during winter. We found that, in winter, glyphosate depressed the growth of S. canadensis, which had viable leaves, but did not affect the growth of winter-dormant native I. cylindrica. These findings suggest that leaf absorption, transportation and function of herbicides depend on physiological activity of plant species at low temperatures (Devine and Bandeen 1983; Klevorn and Wyse 1984; Caplan et al. 2018). Glyphosate may still be transported downwards to the rhizomes, adventitious buds and roots of S. canadensis in winter, become effective belowground and impair regrowth in spring. Studies have indicated that the processes involved in herbicide functioning can be highly affected by temperature (Devine and Bandeen 1983; Klevorn and Wyse 1984). Therefore, the effects of temperature on the efficacy of S. canadensis control should be examined in future studies. The suppressive effects of glyphosate on the growth of S. canadensis were less pronounced in the field experiment than in the pot experiment, very likely due to the much larger plant size. This suggests that the application of a sufficient dosage of glyphosate or repeated annual application is essential for more efficient control of S. canadensis in the field.

Although the growth of *I. cylindrica* plants was strongly suppressed by the presence of S. canadensis when glyphosate was not applied, it accumulated equal biomass in the glyphosate treatments compared with the control treatment (without glyphosate and competition) in winter. This suggests that glyphosate alleviated the competition stresses of S. canadensis on I. cylindrica, owing to the high sensitivity of S. canadensis to glyphosate in winter. Unexpectedly, we did not observe any growth depression in S. canadensis in the presence of I. cylindrica in the glyphosate treatments in winter, either in the pot or field experiments. The suppression effects on the invader by natives induced by herbicide application in winter may occur only after a long period of growth recovery from the disturbances, rather than in the current short observation period, as stresses normally weaken competition intensity, which can recover if stresses recede, as predicted by the stress-gradient hypothesis (Maestre et al. 2009). Studies have shown that the effectiveness of different approaches for invasive species control has emerged at different times (Mahmood et al. 2018), indicating that studies with realistic treatments at large timescales are important for understanding control effects (Sherrill et al. 2022). Therefore, future studies should focus on the long-term effects of spraying glyphosate and other herbicides on S. canadensis during winter.

One caveat is that we recorded two winter active native forbs, *A. lavandulaefolia* and *R. japonicus*. Although these forbs sometimes grow together with *S. canadensis*, unlike most winter-dormant grass species, they normally have distinct spatial separa-

tion from *S. canadensis*, indicating mutual exclusion of these phenologically similar species. Therefore, they are less likely to be injured if herbicide drift is minimised. On the other side, common winter dormant grasses, such as *P. australis*, *I. cylindrica* and *M. sacchariflorus*, tend to form large monocultural patches and are readily replaced by *S. canadensis* (Ye et al. 2019). In the early and middle stages of invasion, herbicide application in winter can selectively control the invader while protecting these dormant natives, which may reverse the succession direction and promote restoration of native cover. The efficiency of winter application of glyphosate and other herbicides in controlling *S. canadensis* while promoting restoration of diverse native plant cover should be examined under the subtropical climate conditions in the future.

We conclude that contrasting winter leaf phenology of the invasive *S. canadensis* and co-occurring native species provides the opportunity to control *S. canadensis* with glyphosate in winter, resulting in higher herbicide selectivity and efficiency under the subtropical climatic conditions. The principles behind this approach may be generally applicable in situations where invasive plant species and their co-occurring native species show distinct phenological niche separations.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualisation, X.Y, M.W, R.Z and F.Y; methodology, X.Y.; data curation and investigation, X.Y, J.M, J.L and R.M.; writing—original draft preparation, X.Y; writing—review and editing, X.Y, M.W, R.M. and F.Y; supervision, M.W. and F.Y.; project administration, X.Y. and M.W.; funding acquisition, X.Y. and M.W. All the authors have read and agreed to the published version of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

The mean daily maximum air temperature, mean daily minimum air temperature and mean monthly precipitation of Fuyang, Hangzhou over the last ten years (2014–2023)

Authors: Xiaoqi Ye, Jinliu Meng, Ruixiang Ma, Jili Liang, Ming Wu, Rongzhou Man, Feihai Yu Data type: tif

Explanation note: The data were edited from the China Meteorological Data Service Center (http://data.cma.cn/).

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Supplementary material 2

Mean daily air temperature of Fuyang, Hangzhou from 1 March 2022 to 28 February 2023

Authors: Xiaoqi Ye, Jinliu Meng, Ruixiang Ma, Jili Liang, Ming Wu, Rongzhou Man, Feihai Yu Data type: tif

Explanation note: The data were edited from China Meteorological Data Service Center (http://data.cma.cn/).

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Research Article

Evaluation of climate conditions and ecological traits that limit the distribution expansion of alien *Lolium rigidum* in Japan

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Abstract

Invasive alien plants cause severe global problems; therefore, determining the factors that lead to the success or failure of invasion is a critical question in the field of invasion ecology. In this study, we aimed to determine the factors underlying differences in the distribution range of alien plants in Japan by investigating why Lolium multiflorum thrives in a wide range of habitats while L. rigidum is mainly distributed on sandy beaches. We initially evaluated environmental niche suitability through species distribution modelling and subsequently examined whether species traits influence the differences in range expansion between the two species. We used MaxEnt modelling to identify potential environmental niches for both species. The analysis revealed that L. rigidum was considerably less suited to the Japanese climate compared to L. multiflorum, with high summer precipitation in Japan identified as one of the climatic factors limiting the distribution of L. rigidum. Given that these winter annual plants remain dormant as seeds during summer, in subsequent experiments, we buried seeds in paddy field soil and sandy beach sand during summer and evaluated their survival rate in autumn. The survival rate of L. rigidum seeds was significantly lower than that of L. multiflorum, particularly in paddy soil. Factors contributing to seed mortality may include the decay or early germination of L. rigidum seeds under Japan's high rainfall conditions. This study emphasises the importance of considering local environmental factors alongside climate niche modelling in the risk assessment of invasive species. Moreover, the integration of species distribution modelling for large-scale evaluations and manipulation experiments for fine-scale assessments proved effective in identifying climatic conditions and species traits influencing the success or failure of alien species invasion.



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Copyright: © Kentaro Uehira & Yoshiko Shimono. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). **Key words:** Coastal environment, invasion success, local environmental condition, MaxEnt, seed burial experiment, seed decay, species distribution modelling, summer rainfall

Introduction

Invasive alien plants cause issues such as loss of biodiversity, reduced crop yield, and health risks (Vitousek 1990; Pyšek and Richardson 2010; Vilà et al. 2010). However, not all introduced species become invasive in a new habitat. It has been hypothesised that only about 10% of species successfully progress through the consecutive steps of the invasion process. Specifically, approximately 10% of species transported beyond their native range will be released or escape in the new regions, about 10% of these introduced species will successfully establish themselves, and about 10% of established species will become invasive (tens rule; Williamson and Fitter 1996). Therefore, extensive long-term research has been conducted to

identify which environments facilitate the establishment of invasive species and which species' characteristics contribute to the successful establishment (Richardson and Pyšek 2006; Hayes and Barry 2008; Hui et al. 2016).

The impact of climate on determining invasion success or failure has been extensively investigated using the species distribution modelling (SDM) approach. This approach can estimate suitable climate conditions for a target species based on historical climate and species occurrence data and predict the distribution suitability of the species across geographic and temporal scales. This approach has revealed that specific climate factors can influence the potential geographic distribution of a species (Phillips et al. 2006; Phillips and Dudík 2008; Wiens et al. 2009). SDM is widely used in the risk assessment of invasive species, largely because the climatic niche of invasive species in their new destination areas often resembles that in their source areas (Liu et al. 2020). Although SDM is a robust tool that combines statistical modelling and geographic information systems to gain insights into the potential environmental factors influencing species distributions (Phillips et al. 2004), it cannot definitively establish causal relationships between environmental factors and species distribution (Merow et al. 2013). To identify the drivers of invasion processes in destination areas, experiments manipulating environmental factors are necessary; however, few studies have combined both SDM and experimental manipulation approaches.

The genus *Lolium* includes two outcrossing annual species: *L. multiflorum* Lam. and *L. rigidum* Gaudin, which are native to the Mediterranean region (Terrell 1968). They have been introduced as a forage crop and turfgrass in numerous countries across the globe (Humphreys et al. 2010), resulting in their escape from controlled areas and becoming problematic weeds. *L. multiflorum* is widely distributed throughout Europe, North America, South America, northern and eastern Africa, Australia, Central Asia, and eastern Asia (GBIF 2022a) (Fig. 1a). It directly reduces crop yield by spreading as a weed in agricultural fields (Liebl and Worsham 1987; Sønderskov et al. 2020) and serving as a habitat for rice-ear bugs, which are important pests of rice (Yoshioka et al. 2011). *L. rigidum* is distributed throughout Europe, Australia, North America, South America, South Africa, East Asia, and West Asia (GBIF 2022b) (Fig. 1b). This species has developed tolerance to multi-



Figure 1. The distribution of A *L. multiflorum* and B *L. rigidum*. This distribution map was made using the occurrence data from GBIF (GBIF 2022a, 2022b).

ple herbicides, posing a serious challenge (Heap 2023), particularly in wheat cultivation in Australia (Owen et al. 2014). In Japan, *L. multiflorum* has predominantly been introduced as forage and revegetation materials but has spread as weeds in universal environments such as farmland and riverbanks. Conversely, *L. rigidum* has mainly been introduced to Japan through imported grains contaminated with *L. rigidum* seeds and subsequently became established (Shimono et al. 2015; Higuchi et al. 2017; Hirata et al. 2023). Originally a weed in agricultural fields, it has only been specifically established in local environments, such as sandy beaches, in Japan (Hirata et al. 2023).

In this study, we aimed to investigate environmental factors and species traits that contribute to the distribution expansion of alien plants in Japan using both SDM and manipulation experiments, focusing on congeneric species of the genus *Lolium* with different distribution ranges in Japan. Specifically, we investigated why *L. multiflorum* thrives in a wide range of habitats whereas *L. rigidum* has not been able to spread to agricultural fields. We initially examined the suitability of the climate in Japan for the spread of these *Lolium* species using the MaxEnt model (Phillips et al. 2006), which is the most common SDM approach. MaxEnt analysis revealed summer precipitation as a key climatic factor limiting the distribution of *L. rigidum* in Japan. In addition, as *L. rigidum* and *L. multiflorum* are winter annuals that exist as seeds during the summer, we evaluated the seed survival rates of both *Lolium* species in the soil from summer to autumn.

Methods

Species distribution modelling

Global distribution data for *L. multiflorum* and *L. rigidum* were acquired from the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/) in July 2022 (GBIF 2022a, 2022b). Although the GBIF data contained accurate information on the coordinates, we used all the data without specifying it. This approach helps us avoid the risk of simultaneously losing data from specific regions that would have been lost had we specified the accuracy of the coordinates. As environmental data, we used 19 bioclimatic variables worldwide derived from temperature and precipitation records from 1970 to 2000 (See Suppl. material 1: table S1), sourced from the WorldClim database (https://www.worldclim.org/) (Fick and Hijmans 2017). This dataset is frequently used for ecological studies focusing on SDM (Booth et al. 2014). The raster data resolution was set to 10 min. Duplicated occurrence points were eliminated in the modelling process, resulting in one occurrence point per cell. As a result, 4,099 and 9,040 points were retained for *L. rigidum* and *L. multiflorum*, respectively.

According to Phillips (2008), 10,000 'background' (or 'pseudo-absence') points are typically sufficient for MaxEnt modelling. However, the number should be sufficient to adequately account for the range of climate variability in the study area, and > 10,000 points may be needed for a large number of occurrence records. In accordance with this recommendation, the background for this study, involving an expansive area and a large number of occurrence records, was set at 30,000 for *L. rigidum* and 50,000 for *L. multiflorum.* Model performance can be further improved by restricting the occurrence of background points to fractions containing occurrence points (Phillips 2008; Anderson and Raza 2010). Therefore, we limited the occurrence of background points to locations within a radius of 500 km from the occurrence points of *Lolium* species.

We implemented variable selection in the subsequent steps because of multicollinearity among environmental variables potentially decreasing prediction accuracy (Heikkinen et al. 2006; Dormann et al. 2013). Firstly, we calculated the Pearson correlation matrix for all combinations of variables. Secondly, we utilised the MaxEnt v.3.4.3 software to run a MaxEnt model with all 19 variables. Then, we extracted variable pairs with a correlation coefficient > 0.7, as per the criteria set by Green (1979). The variable with a smaller contribution to the MaxEnt model was then removed based on the variable importance of MaxEnt output. Finally, the MaxEnt model was reconstructed using only the remaining variables. We conducted five-fold cross-validation and assessed goodness of fit using the area under the receiver operating characteristic (ROC) curve (AUC) value, which ranges from 0 to 1; a value of 0.5 indicates random guessing, whereas a value of 1 signifies perfect classification (Fielding and Bell 1997). A response curve was created for each variable (meaning that a predictive model was created for each variable), and the contribution of each variable to the model was evaluated using a jackknife test (both are built-in functions of the MaxEnt software). The response curve was overlaid with the density distribution of the corresponding climate variable in Japan. Other MaxEnt settings were set as default.

To identify the environmental variables impacting the suitability of *Lolium* species in Japan, we set one Japanese climate variable to the optimal value determined from MaxEnt output response curves while keeping the remaining variables at their original values to simulate changes in distribution suitability. MaxEnt was performed for each value-adjusted variable using the same parameter settings as mentioned above.

Seed burial experiment

L. rigidum and *L. multiflorum* are both winter annuals, germinating in autumn, flowering in spring, and dispersing seeds in early summer. Therefore, seed burial experiments were performed to assess their survival rates from summer to autumn. In June 2021, mature seeds of *L. rigidum* and *L. multiflorum* were collected from naturalised populations along a sandy beach (34.7923°N, 136.558°E) and the levee of a paddy field (34.7991°N, 136.5342°E) in Mie Prefecture. Paddy soil consisting mainly of clay was collected from the experimental field at Kyoto University (35.0321°N, 135.7835°E) and beach soil was collected at 5-cm depths on seven sandy beaches in central Japan (Suppl. material 1: table S2), where *L. rigidum* growth was confirmed. We used sandy beach soils collected from multiple locations because the sand grain size varies depending on the location.

Fifty seeds of each species were packed into non-woven fabric bags (length: 9 cm; width: 7 cm) that also contained 5.0 g of autoclaved paddy soil or beach sand to prevent the seeds from adhering to each other. One bag containing each species was buried at depths of 7 cm and 15 cm in plastic pots (diameter: 16.8 cm; height: 19.8 cm) filled with paddy soil and beach soil in July 2021, respectively. There were three and seven replications per species for beach soil and paddy soil, respectively. Intense sand movement by strong wind in sandy beaches and tillage in paddy fields results in seeds being buried at varying depths. Therefore, to investigate whether differences in burial depth affect survival rates, two burial depths

were set in this study. The plastic pots were placed on the experimental field at Kyoto University, remained exposed to rainfall, and then retrieved in October of the same year, coinciding with the germination period under natural conditions. The precipitation from July to October 2021 was 390 mm (July), 468 mm (August), 180 mm (September), and 41 mm (October) (Japan Meteorological Agency 2024).

Additionally, in 2024, similar experimental setups were conducted using paddy soil from Kyoto University and sandy beach soil collected in Mie Prefecture (34.7923°N, 136.558°E), measuring soil moisture contents (%) from June to July using digital handheld moisture meter (PMS-714, Omega Engineering inc.).

Seeds were collected from the soil, and those without hard embryos were discarded. Traces of rooting were checked. The remaining seeds were placed on 9.0-cm Petri dishes and germinated in an incubator (LH-30-8CT, Nippon Medical & Chemical Instruments) at 30/20 °C with alternating 12/12 h cycles (i.e., 12 h light and 12 h dark) for a week. These conditions were based on Rodriguez et al. (1998). Finally, we used the 2,3,5-triphenyltetrazolium chloride (TTC) method to differentiate between dormant and dead seeds with ungerminated, hard embryos. The seeds were bisected to expose the embryo, then immersed in a 1% TTC solution, and incubated overnight at 25 °C in the dark. The following morning, we examined the seeds to determine whether the embryos had been stained. The total number of viable seeds during the burial period was calculated by summing the number of individuals that had already germinated in the soil (but were alive at the time of retrieval), those that germinated in the incubator, and those that were stained by the TTC method. The total number of dead seeds was calculated by summing the number of individuals that lacked hard embryos (including those that showed traces of germination and had already died) and those that were not stained by the TTC method.

A hierarchical linear model with binomial errors was employed to evaluate the survival rate of *Lolium* spp. seeds. The primary effects examined in this study were *Lolium* spp., soil type (paddy or beach soil), and burial depth, whereas the random effects were plastic containers and beach soil collection sites. Statistical analysis was performed using the rstan package (Stan Development Team 2024) in R ver. 4.2.3 (R Core Team 2023).

Results

Species distribution modelling

After variable selection, eight variables remained for *L. rigidum*: mean diurnal range (bio2), temperature seasonality (bio4), mean temperature of the wettest quarter (bio8), mean temperature of the warmest quarter (bio10), precipitation seasonality (bio15), precipitation of the driest quarter (bio17), precipitation of the warmest quarter (bio18), and precipitation of the coldest quarter (bio19). Similarly, seven variables remained for *L. multiflorum*: annual mean temperature (bio1), mean diurnal range (bio2), temperature annual range (bio7), mean temperature of the wettest quarter (bio8), precipitation of the coldest quarter (bio13), precipitation seasonality (bio15), precipitation of the coldest quarter (bio19) (See Suppl. material 1: table S3 for correlation coefficients and Suppl. material 1: table S4 for variable contributions).

The average AUC values were 0.77 and 0.82 for *L. rigidum* and *L. multiflorum*, respectively. These values are considered moderately predictive according to the AUC criteria described by Vanagas (2004). The jackknife test showed that the most useful and unique information for predicting the global distribution was temperature seasonality (bio4) for *L. rigidum* and annual mean temperature (bio1) and temperature annual range (bio7) for *L. multiflorum* (Fig. 2).

Based on the MaxEnt model of *Lolium* species projected for Japan, the mean probability of *L. rigidum* presence was 0.055 (SD: 0.035), whereas that for *L. mul-tiflorum* was 0.31 (SD: 0.076) (Fig. 3).

Fig. 4 displays the response curves and density distributions of both *Lolium* species in Japan for each bioclimatic variable. Japanese climate varies considerably from the optimum ranges for *L. rigidum* in terms of temperature seasonality (bio4) and precipitation of the warmest quarter (bio18) and for *L. multiflorum* in the mean temperature of the wettest quarter (bio8).

When Japanese bioclimatic variables were fixed to the values that maximise suitability for *L. rigidum*, which had a particularly low probability of presence in Japan, in the response curves, changes in mean diurnal range (bio2), mean temperature of the wettest quarter (bio8), mean temperature of the warmest quarter (bio10),



Figure 2. Relative predictive power of each bioclimatic variable based on the regularised training gain in MaxEnt models, as estimated using the jackknife test, for A *L. rigidum* and B *L. multiflorum*.



Figure 3. Probability of presence in Japan estimated by MaxEnt for A L. rigidum and B L. multiflorum.



Figure 4. A response curves for *L. rigidum* (red lines and blue shades) and density distributions of Japanese bioclimatic variables (green histograms) **B** response curves for *L. multiflorum* (red lines and blue shades) and density distributions of Japanese bioclimatic variables (green histograms). The horizontal axis displays the variation range of the bioclimatic variables. The first vertical axis shows the predicted suitability of the target species, while the second vertical axis shows the density distribution of Japanese bioclimatic variables. The red line represents the mean of the five iterations of the estimation, while the blue shade indicates its standard deviation.



seasonality of precipitation (bio15), precipitation in the driest month (bio17), and precipitation in the coldest month (bio19) had minimal impact on the probability of the presence in Japan, with mean values of 0.055, 0.058, 0.055, 0.051, 0.046, 0.058, respectively (Suppl. material 1: fig. S1). Conversely, temperature seasonality (bio4) and precipitation of the warmest quarter (bio18) increased the probability of presence to mean values of 0.28 (SD: 0.038) and 0.26 (SD: 0.14), respectively (Fig. 5).



Figure 5. Probability of presence estimated by Maxent for *L. rigidum* when the bioclimatic variables fixed at their optimum values **a** shows bio04 and **b** shows bio18.

Seed burial experiment

The diurnal variation of soil moisture contents for 1 month (from June 22nd to July 20th, 2024) revealed that the soil moisture of beach soil decreased rapidly in the absence of rain, and, on all days, the soil moisture was higher in paddy soil than in beach soil (Fig. 6). An average of 94% (SD: 4.1%) of seeds were retrieved from soil-filled bags. The average seed survival rates of *L. rigidum* and *L. multiflorum* were 63% and 79%, respectively (Fig. 7). Hierarchical linear model results showed that *L. rigidum* had a significantly lower seed survival rate than that of *L. multiflorum* and that the seed survival rate was significantly higher in sandy beach soils than that in paddy soils, with a minor impact of burial depth (Fig. 8a). The model-estimated survival rates of both *Lolium* spp. at different depths in paddy and beach soils indicated that the seed survival rate of *L. rigidum* in paddy soil was the lowest among all combinations, dropping below 50% (Fig. 8b).



Figure 6. Daily precipitation in Kyoto City (top) and soil moisture content (bottom) from June 22th to July 20th.



Figure 7. Breakdown of seeds retrieved from soil-filled bags. PDY represents paddy soil, whereas the remaining seven symbols indicate the collection sites of sandy beach soil samples. For further details, refer to Suppl. material 1: table S2.



Figure 8. A estimated coefficients for survival rates (median and 95% estimate interval) of *L. rigidum* compared to *L. multiflorum* (upper), sandy beach soil compared to paddy soil (middle), and burial depth of 15 cm compared to 7 cm (lower) on a logit scale **B** estimated survival rates of *L. multiflorum* (green) and *L. rigidum* (blue) in each soil and at each depth. Error bars indicate 95% estimate intervals.

Discussion

In the present study, the MaxEnt results indicated that *L. rigidum* was less suited to the Japanese climate than *L. multiflorum*. The Japanese climatic conditions that deviated considerably from the predicted suitability range for *L. rigidum* were temperature seasonality and summer precipitation. Temperature seasonality is a determinant influencing the northern limits of plants and animals in the Northern Hemisphere (Wiens et al. 2006; Qian et al. 2022). *L. rigidum* is found in temperate zones, and as Japan is not located at the northern limit, it is currently unclear what ecological significance can be ascribed to temperature seasonality as a climatic variable that explains the distribution of *Lolium* species in Japan.

MaxEnt predicted that high summer precipitation in Japan renders the environment unsuitable for *L. rigidum*. The burial seed experiment conducted during summer revealed a higher mortality rate for *L. rigidum* seeds than for *L. multiflorum* seeds. Potential reasons for seed mortality in this experiment include seed decay or premature germination. Additionally, in real-world field conditions, various factors such as predation and fungal infections, which were not accounted for in our experiment, could further reduce seed survival rates (Ranganathan and Groot 2023). To better evaluate the fitness, the survival rate of seedlings after germination in each field should be investigated in future studies. Nevertheless, comparing our experimental findings with *L. multiflorum*, which has successfully expanded its distribution across a wide range of environments, suggests that seed decay due to heavy summer rains likely reduces the fitness of *L. rigidum* in the field.

Water availability is the primary limiting factor for terrestrial plant production (Lambers and Oliveira 2019), and soil hydrological properties at a fine scale effectively determine plant distribution (Silvertown et al. 1999). As sandy beaches are generally arid environments (Brakenhoff et al. 2019), our experimental results confirmed that under the same precipitation conditions, soil moisture content was lower in sandy soil than in paddy soil. This lower moisture content suggests that seeds of *L. rigidum* are less likely to decay in such areas, and consequently, the establishment of *L. rigidum* in Japan is possibly locally limited to sandy beaches.

L. rigidum is native to the Mediterranean region (Terrell 1968) and has emerged as a major weed problem, especially under the Mediterranean-type climate of Western Australia (Owen et al. 2014). Japan, with its monsoon climate, receives considerably higher precipitation than the aforementioned regions (annual precipitation of 1,668 mm/year in Japan versus 733 mm/year in the Perth metropolitan area, Australia) (Commonwealth of Australia 2023; The World Bank 2023). In addition, as a consequence of the widespread cultivation of rice paddies throughout the country, the soil moisture content in Japanese agricultural land is generally high. Given this context, seed decay can be considered a weed control method in Japan (Kida and Asai 2006; Aoki et al. 2012). Although the drought sensitivity and flood tolerance of seedlings have been previously assessed to understand plant distribution patterns (Engelbrecht and Kursar 2003; Jansen et al. 2005), seed moisture tolerance has mostly been neglected and underestimated.

Sandy coasts are typically arid, nutrient-poor, and highly susceptible to salt spray, sand deposition, and strong winds, all of which are limiting factors for plant establishment (Maun 1994). Dryland salinity is a major problem in agricultural areas in Australia (Briggs and Taws 2003; George et al. 2006), and *L. rigidum*, a weed that thrives in such environments, may be highly tolerant to drought and

salinity stress. Weeds that have become problematic in arid agricultural areas, not only in Australia, may have the potential to establish themselves on sandy beaches in wetter areas because of their drought and salt tolerance.

In the present study, niche modelling solely based on climate variables indicated that *L. rigidum* was not well-suited for distribution in Japan; however, it is actually expanding its distribution on sandy beaches. This suggests that climate niche modelling is insufficient for fine-scale predictions and underestimates the invasion risk of alien species in some specialised habitats. When applying SDM to finer scales, local predictors, such as soil conditions and topography, must be considered (Pearson and Dawson 2003). However, acquiring these variables over a large spatial extent is challenging (Bradley et al. 2012).

In summary, we conducted large-scale niche modelling to identify environmental factors predicted to limit the distribution of invasive species. Subsequently, we examined whether these factors actually affect the fitness of these species through manipulative experiments. Although manipulative experiments alone cannot account for all factors, focusing on environmental factors suggested by niche modelling and estimating the causality of invasion success through these experiments are highly valuable.

Conclusions

In this study, we focused on the alien *L. rigidum*, which has only expanded locally on sandy beaches in Japan, and *L. multiflorum*, which has been successfully established across various environments in Japan. We investigated the limiting factors for the distribution expansion of *L. rigidum*. Through manipulative experiments, we found that *L. rigidum* had a higher seed mortality rate, especially in paddy soil, than that of *L. multiflorum*. This result aligns with a suggestion from SDMs that the summer rainfall in Japan may be excessive for *L. rigidum*.

Predictions based solely on climate variables using SDM revealed that *L. rigidum* is not suitable for the Japanese environment. However, *L. rigidum* is actually expanding its distribution on Japanese sandy beaches. This indicates that niche modelling based on specific climate variables alone may underestimate the invasion risk of alien species. The combined use of large-scale niche modelling and manipulative experiments, as conducted in this study, demonstrates the importance of this approach for assessing the invasion risk of species in both regional and local environments.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Kentaro Uehira: Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing - original draft. Yoshiko Shimono: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review and editing.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary information

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Research Article

An ecoregion-based approach to evaluate invasive plant species pools

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Abstract

Invasive alien species are an important component of global change, threatening biodiversity, ecosystem functioning, economy, and human health. The number of alien species that attain the invasive status has experienced an exponential increase in recent years, leading some government agencies and stakeholders to allocate substantial resources to early detection, control, mitigation, and eradication programs. To develop effective nature conservation strategies, it is crucial to understand the invasive status of alien species and to identify priority species for management at spatial scales with a biogeographical basis. Despite significant progress in producing lists of alien species at the country level, a standard methodology for species assessment within ecological regions (i.e., regions with similar environmental or biogeographical characteristics) is still lacking. Here, we develop a systematic approach to determine invasion status and to prioritize invasive alien plant species within an ecoregion. We apply this approach in the Cantabrian Mixed Forests ecoregion, which encompasses biogeographically related areas from N Portugal, NW Spain, and SW France, and is strongly affected by plant invasions. By combining scientific evidence with expert opinion on the ecological characteristics of alien plants, we identified 175 invasive plant species in the study ecoregion, of which 37 cause massive environmental and/or socio-economic impacts. For each species, we provide comprehensive information and recommendations for scientists, land managers, policy makers, and other stakeholders under a biogeographical basis. This information includes species characteristics, invasion status/ level, population trends, geographic locations and range size, local abundance, environmental and socio-economic impacts, and invaded habitats. We also accounted for administrative divisions within the ecoregion to facilitate the use of such evaluations in local-scale management and conservation plans. Our framework may be applied to any ecoregion worldwide, enhancing the assessment and management of invasive species pools within biogeographically meaningful regions.

Key words: Cantabrian Mixed Forest ecoregion, Iberian Peninsula, invaded habitats, invasive alien plant species, invasion level, invasion status, invasive alien plant species, invasive species impacts, management



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Introduction

Invasive alien species are an important factor associated with global change, causing multiple ecological, economic, and social impacts around the world (Vilà et al. 2011; Shackleton et al. 2019; Diagne et al. 2021). In the IPBES Regional Assessments and Global Assessment Report, invasive alien species were identified as one of the main leading causes of biodiversity loss worldwide (IPBES 2019, 2023). The challenge of understanding, preventing, and early detection of biological invasions is a key priority for strategies and action plans adopted by countries and organizations to tackle key drivers of biodiversity loss and to promote more sustainable and integrated environmental management (e.g., the European Union's Biodiversity Strategy for 2030, the Post-2020 Framework of the Convention on Biological Diversity, and the United Nations 2030 Agenda for Sustainable Development). Understanding the invasion status of alien species established in a territory and identifying priority species for management is, therefore, pivotal to improve conservation efforts in natural and semi-natural habitats.

Despite significant progress in producing lists of invasive alien species for individual countries (Sanz-Elorza et al. 2004; Essl et al. 2011; Gederaas et al. 2012; Marchante et al. 2014; Pergl et al. 2016), a standard methodology for the assessment of invasive alien species within ecological regions is lacking in the literature. Ecological regions (ecoregions, hereafter) are ecologically homogeneous units that occur within a country or across several countries (see for example Ecoregions2017 ©Resolve). The geographic distribution of ecoregions is related to abiotic (e.g., climate and soil) and biotic (e.g., dominant vegetation) factors, and they are assumed to encompass areas with a similar biogeographic history and recurrent local ecosystems (Bailey 2004). The importance of ecoregions in environmental assessment and management has long been recognized (Omernik 2004), providing a holistic framework for integrating research and management actions in relatively large geographical areas (Loveland and Merchant 2004). Since ecoregions are effective units for biodiversity studies and nature conservation, they should also be preferred as study areas for evaluating invasive alien species. Managing invasive species at the ecoregion scale has the advantage of generating information and applying management actions throughout ecologically homogeneous areas, which are likely to be invaded by species with similar ecological characteristics, i.e. to share invasive species pools. The invasive behavior of a given species in part of an ecoregion may be a good predictor of its invasive potential throughout the ecoregion. This ecoregion-based approach may improve current efforts to manage invasive species within and across political divisions. Assessments at the jurisdictional scale are more feasible to implement, but may only consider part of an ecoregion, excluding information from nearby ecologically similar areas, which could be key for control efforts. Thus, developing a method to prioritize trans-boundary management based on a biogeographical basis is important to tackle biological invasions.

Species invasion within ecoregions is a seral process that involves overcoming biotic and abiotic filters at local and landscape scales (Theoharides and Dukes 2007; Catford et al. 2009). After surpassing major biogeographical barriers (e.g., mountain ranges and oceans), alien species (a.k.a. introduced, exotic, non-native) must survive and form self-sustained populations to continue the invasion process within an ecoregion (naturalization stage; Catford et al. 2009; Fig. 1). Then, the naturalized species must produce reproductive offspring (either by seeds or other



Figure 1. The generalized species invasion curve, adapted from Harvey and Mazzotti (2014) and Haubrock et al. (2022). Labels at the top refer to the stages of the invasion process (the invasion stage is divided into low, medium, and high level). Bold labels refer to management actions appropriate at each stage of invasion. White boxes below the graph indicate terms used to refer to alien species within each invasion stage. Economic return values listed at the bottom indicate the amount of money that is returned based on the money invested at each invasion stage, adapted from Victorian Government (2010) and Reid et al. (2021).

propagules), often in very large numbers, at considerable distances from the parent plants to be considered as invasive (Richardson et al. 2000; Pyšek et al. 2004). Such invaders can cause negative environmental, social, and/or economic effects within the ecoregion.

Once the alien species reaches the status of "invasive" within an ecoregion, its population density and range size progressively increase, at least until they fill a certain niche. Invasive species generally start with a small number of localized populations, whose eradication may be feasible, as represented in the widely accepted invasion curve (Harvey and Mazzotti 2014; Robertson et al. 2020; Haubrock et al. 2022; Fig. 1). However, invasive species are expected to rapidly increase in distribution and abundance throughout an ecoregion, because the specific environmental conditions of invaded areas (i.e., suitable areas) are spatially recurrent (Bailey 2004), thus, facilitating the invasion process. At this stage, the eradication of the species becomes unlikely, and managers should change their goals to limit further spread across the ecoregion with containment measures (Robertson et al. 2020). Finally, invasive species become widespread and abundant throughout the ecoregion, requiring long-term management aimed at population suppression and resource protection (Harvey and Mazzotti 2014; Haubrock et al. 2022). This control of invasive species should consider restoration measures, in an adaptative management approach (IPBES 2023). It is important to note that the invasion levels and management objectives described here (and depicted in Fig. 1) only apply to invasions in terrestrial and closed water systems, but not to marine and connected water systems (IPBES 2023).

Resources used for environmental management are often limited. The successful management of invasive species is often constrained by insufficient or inconsistent

funding and limited public awareness (Januchowski-Hartley et al. 2011). Thus, action plans generally consider which species should be targeted first, based on their distribution, local abundance, and environmental and socio-economic impacts on natural and semi-natural habitats (Pergl et al. 2016; Fristoe et al. 2021). Invasive species prioritization requires both quantitative data and expert knowledge based on scientific evidence that should be preferably related to a territory with similar environmental characteristics, considering both geographic and ecological features (i.e., an ecoregion).

In this study, we develop a systematic approach to determine the invasion patterns of the current invasive plant species and to identify priority species for management at the ecoregion level (Fig. 2). We applied this approach in the Cantabrian Mixed Forests, a European ecoregion severely affected by plant invasions. We combined published data with expert knowledge to 1) create a comprehensive list of the invasive alien plant species pool for the ecoregion, 2) provide information on invasion status/level (i.e., low, medium, and high levels of invasion), population



Figure 2. Proposed systematic approach to evaluate the invasive plant species pool of an ecoregion and to identify priority species for driving management actions.
trends, range size, and local abundance of each species, and 3) identify priority species based on their environmental and socio-economic impacts, and the type of invaded habitats.

Materials and methods

Study ecoregion

The study was conducted in the Cantabrian Mixed Forests ecoregion (Fig. 3) as defined by the World Wide Fund for Nature (WWF), and described in the RESOLVE Ecoregions dataset (Ecoregions2017 @Resolve; https://ecoregions. appspot.com/). We slightly re-defined the ecoregion limits to accommodate the latest biogeographical updates in the Iberian Peninsula and SW France (Fernández-Prieto et al. 2020). The extent of the ecoregion largely fits with the Atlantic-European Province in the Iberian Peninsula (Rivas Martínez et al. 2011) and the Natura 2000 Atlantic biogeographic region in the Iberian Peninsula (https:// ec.europa.eu/environment/nature/natura2000/platform/faq/index_en.htm). The ecoregion includes territories in north-western Portugal, north-western Spain, and a small area in south-western France, representing c. 20% of the Iberian Peninsula (Fig. 3). Administratively, the study ecoregion comprises the Spanish autonomous regions of Asturias, the Basque Country, Cantabria, Galicia, and northern parts of Navarra and Castilla y León (including the provinces of Zamora, León, Palencia, and Burgos), as well as the Portuguese provinces of Aveiro, Braga, Bragança, Porto, Viana do Castelo, Vila Real, and Viseu, and the south-western part of the French Nouvelle-Aquitaine region.

Unlike most of the Iberian Peninsula, this ecoregion provides optimal conditions for warm-temperate and humid ecosystems, with precipitation patterns determined largely by the frequency of Atlantic fronts from the northwest. Such climatic variation, together with its varied topography, soil types, and land uses, fosters a high diversity of ecosystems, being one of the most important areas for terrestrial biodiversity, carbon, and water conservation of the Iberian Peninsula (Jung et al.



Figure 3. A ecoregions of western Europe, obtained from the RESOLVE Ecoregions dataset (Ecoregions2017 ©Resolve; https://ecoregions.appspot.com/). The black square includes the study ecoregion (in green), and part of other ecoregions **B** study ecoregion. Black lines delineate the study ecoregion and divide the different major areas within the ecoregion (see below), whereas yellow lines indicate Spanish autonomous regions and Portuguese provinces. Numbers correspond to major areas within the ecoregion: (1) north-western Portugal, (2) Galicia, western Zamora, and western León, (3) Asturias and northern León, (4) Cantabria, northern Palencia, and north-western Burgos, and (5) Basque Country, north-eastern Burgos, northern Navarra, and south-western French Nouvelle-Aquitaine.

2021). However, the studied ecoregion is also one of the most sensitive areas for biological invasions (Sanz-Elorza et al. 2004; Gassó et al. 2009; Aguiar and Ferreira 2013). In fact, the studied ecoregion is considered a current and future hotspot for plant invasions due to its benign climate and complex international trade connections, especially in the coastline (Gassó et al. 2012; Fernández de Castro et al. 2018). Current efforts to manage invasive species within the ecoregion are mostly conducted within political divisions (e.g., countries, administrative provinces, and regions mentioned above). A cross-boundary collaboration among political divisions (e.g., regions and countries) would likely enhance local and regional action plans aimed at preventing, early detection of, and control of biological invasions.

Identification of the ecoregional invasive species pool

We first searched national and regional reports and databases to gather an initial list of plant species considered as invasive or potentially invasive in the study ecoregion (Sanz-Elorza et al. 2004; Fagúndez and Barrada 2007; González-Costales 2007; Campos and Herrera 2009; Marchante et al. 2021). We also gathered existing legal national lists of invasive species in use by the countries included in our ecoregion: Portugal (Decreto-Lei n.º 92/2019), Spain (Ley 42/2007, Real Decreto 630/2013, Real Decreto 216/2019, Orden TED/1126/2020, Orden TED/339/2023), and France (Inventaire national du patrimoine naturel; https://inpn.mnhn.fr). Then, we critically revised the initial list using a combination of local literature, expert knowledge, and evidence from both scientists and managers to create a complete and uniform (i.e., ensuring that the same definition of invasive was used) checklist of invasive plant species present in the ecoregion, here called the "ecoregional invasive plant species pool". We checked the World Flora Online (http://www.worldfloraonline.org/) and the Kew POWO (https://powo.science.kew.org/) databases to assign the proper taxonomy names and the synonyms for each invasive species.

Alien species are those whose presence in a given region is due to intentional or unintentional human involvement (Richardson et al. 2000; Pyšek et al. 2004). We considered an alien plant species as invasive when it produces reproductive offspring (either by seeds or other propagules) in areas distant from sites of planting/ sowing, without direct intervention by humans, independent of their environmental, economic, and social impacts (sensu Richardson et al. 2000; Pyšek et al. 2004). This definition of invasive species is widely accepted among the scientific community, and separates species dispersal from their impacts (Catford et al. 2009). Those species that cause impacts are usually termed transformers (Richardson et al. 2000; Pyšek et al. 2004; Catford et al. 2009). However, legal definitions, and some authors, use the term invasive to refer to those established alien species that threaten or have adverse impacts on biodiversity and ecosystem services (IPBES 2023; Portuguese "Decreto-Lei n.º 92/2019"; Spanish "Ley 42/2007" and "Real Decreto 630/2013"). To have the most comprehensive list of invasive alien species, we included alien species already producing reproductive offspring in areas distant from sites of introduction in the study ecoregion and that have the potential to cause environmental alterations and socio-economic losses on the ecoregion (or are already causing them). However, we considered the impacts caused by each invasive species to identify priority species for management, as explained below.

We collected descriptive data on the ecoregional invasive species pool from online databases, such as World Flora Online (http://www.worldfloraonline.

org/), Kew POWO (https://powo.science.kew.org/), USDA Plants (https://plants.usda.gov/), and Ecoregions2017 ©Resolve; https://ecoregions.appspot. com/). Descriptive data included: (1) growth form (graminoid or grass-like flowering plant belonging to the families Poaceae, Cyperaceae, and Juncaceae), forb/ herb, vine, shrub, or tree), (2) lifespan (annual, biennial, or perennial), (3) growing environment (terrestrial or aquatic), (4) continent of origin (Africa, Asia, Australia/Oceania, Europe, North America, or South America), and (5) WWF biogeographic realm of origin (Afrotropical, Indo-Malaya, Australasia, Nearctic, Neotropical, or Palearctic).

Evaluation of invasive alien plant species

Invasion status/level and population trends

We divided the ecoregion into five areas corresponding to administrative units to facilitate the assessment of the species by local experts (co-authors of this study) who classified the invasion status/level and population trends of each species within their main areas of expertise. In Spain, the administrative units largely followed the boundaries of Spanish autonomous regions, in some cases merging proximal areas from regions that were not fully included (e.g., several counties from Castilla y León that belong to the study ecoregion). We defined three categories of invasion status/level: low, medium, and high (Fig. 1). Invasion levels were defined based on the distribution and abundance of the invasive species, which are usually correlated at the landscape scale. These invasion levels may be linked to categories of the invasion curve (Harvey and Mazzotti 2014; Haubrock et al. 2022), which shows management actions appropriate at each stage of invasion. It is important to note, however, that the invasion curve concept was not originally intended to be used for invasion level categorization. Low invasion level included those species whose invasion was localized in a few sites (small number of localized populations; eradication possible; e.g., Xanthium spinosum L. and Yucca gloriosa L.). Medium invasion level referred to those instances where several sites have been invaded (increase in distribution and abundance; eradication unlikely; containment may be the most adequate measure; e.g., Tropaeolum majus L.). High invasion level referred to widespread and abundant invasive species such as Cortaderia selloana (Schult. & Schult.f.) Asch. & Graebn. and Robinia pseudoacacia L. (long-term management aimed at population suppression and resource protection).

Population trends indicated the direction of change in the number of individuals or populations for each invasive species. Local experts assessed these population trends using information from invaded areas and their surroundings. We defined four categories of population trends: positive, negative, neutral, and unknown. Positive trends included species whose populations increase by their own means (natural dispersal). There may be human intervention (e.g., planting in gardens, for erosion control, etc.), but the taxon produces new breeding populations far from the plantation area. Negative trends referred to those instances where populations decrease, either by natural means (e.g., decrement of habitat suitability due to climate change) or by human interventions (management). Neutral trends included those species that maintain their populations (neither increase nor decrease). We used the category "unknown" to refer to those species for which there was not available information on population trends.

Species geographic range

We obtained occurrence data for the invasive species pool at 1-km resolution (or higher) from different databases, including global databases such as the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/; downloaded on 1 June 2023) and iNaturalist (https://www.inaturalist.org/; downloaded on 31 May 2023). We excluded records registered before 1950 to include relatively recent occurrence data and to avoid extinct populations. Also, we removed unsuitable data sources from GBIF (i.e., unknown data sources and fossil and living specimens such as plants living in botanical gardens) and iNaturalist (i.e., unverified data, cultivated individuals, and records without photos). We also obtained data from regional and national administration reports (e.g., González-Costales 2007), projects (e.g., LIFE Fluvial, https://www.lifefluvial.eu/; and LIFE Stop Cortaderia, http://stopcortaderia.org/), personal data bases (e.g., Juan Antonio Campos doctoral thesis), and from the Cantabrian (https://www.chcantabrico.es/) and Miño-Sil (https://www.chminosil.es/) hydrographic administrations. This literature search allowed us to build the most comprehensive and up-to-date database of invasive plant occurrences for the ecoregion (available at Mendeley Data repository; DOI: 10.17632/4gtnr58j2b.1). Geographic range sizes for each species were then calculated as the number of occupied 1×1 km cells across the ecoregion and within each major area of the ecoregion. Geographic ranges should not be confused with the above-mentioned invasion status/level, the latter providing a better representation of the species invasion level since it is based on expert-knowledge, and there are many species with missing spatial data at 1-km resolution (or higher).

Species local abundance

Local abundance referred to the average cover of the species in the plant communities where it occurs, as defined by the cover/abundance scale of Braun-Blanquet, the most common scale used in vegetation surveys in the study area. We included average cover, but it is important to note that a species can reach different abundances depending on the type of invaded habitat. High abundance included those species with >50% of estimated average cover, based on expert knowledge ("4" and "5" categories of the Braun-Blanquet scale). Medium abundance included species with an average cover of 5–50% ("2" and "3" categories of the Braun-Blanquet scale). Low abundance included species with <5% of average cover ("r", "+", and "1" categories of the Braun-Blanquet scale).

Prioritization of invasive alien plant species for management

We identified priority species for management based on their environmental and socio-economic impacts, as well as the type of invaded habitats.

Environmental impacts

Environmental impacts on natural and semi-natural habitats were assessed based on the unified classification of impacts by Blackburn et al. (2014). Environmental impacts were assigned based on expert judgment into three levels: 1) limited/ minimal (unlikely to have caused deleterious impacts on the native biota or abiotic environment), 2) moderate (causes declines in the population densities of native species, but no changes to the structure of communities or to the abiotic or biotic composition of ecosystems), and 3) massive (leads to the replacement and local extinction of native species, and produces changes in the structure of communities and the abiotic or biotic composition of ecosystems; Blackburn et al. 2014).

Socio-economic impacts

Socio-economic impacts on natural, semi-natural, and anthropogenic habitats were classified based on expert judgment into three levels: limited/minimal, moderate, and massive, based on the magnitude of their impact on agriculture, infrastructure, landscape (visual), and human health (Pergl et al. 2016).

Type of invaded habitats

We identified which habitats were invaded by each species, based on expert knowledge. We used the European EUNIS habitat classification (Chytrý et al. 2020) to differentiate broad habitat categories: coastal habitats (marshes, dunes, beaches, and cliffs), wetlands (peatlands, bogs, and water bodies), grasslands (lands dominated by grasses, forbs, mosses, or lichens), shrublands (heathlands, scrub, and tundra), forests (forests and wooded lands), vegetated man-made habitats (crops, gardens, roadsides, hedgerows, and plantations), and constructed/industrial habitats (human settlements, buildings, and industrial developments).

Priority scores

Each invasive species was assigned a score for the above-described categories: environmental impacts, socio-economic impacts, and type of invaded habitats. Environmental and socio-economic scores (separately) corresponded to 0 (limited/minimal impact), 0.5 (moderate impact), and 1 (massive impact). The score for the type of invaded habitats corresponded to 0 (species invading only man-made habitats such as vegetated man-made habitats and constructed/industrial habitats) and 1 (species invading natural habitats such as coastal habitats, wetlands, grasslands, shrublands, or forests).

Final priority scores ranged from 0 to 3 and corresponded to the sum of individual scores assigned to environmental impacts, socio-economic impacts, and type of invaded habitats. We categorized the invasive species into three prioritization categories, based on their priority scores: low (0 to 1), medium (1.5 to 2), and high (2.5 to 3) priority species.

Results

Ecoregional invasive species pool

We identified a total of 175 invasive plant species from 49 families in the WWF Cantabrian Mixed Forests ecoregion (Table 1; Suppl. material 1), which corresponds to approximately 4–5% of the ecoregional flora. Species considered as invasive in previous regional lists, but which were excluded in our invasive species pool, are shown in Suppl. material 2, along with the reason for their exclusion (i.e., naturalized species, archaeophytes, or even native species). Over half of the

species (55%) identified as invasive are included in the legal national lists of the countries that are part of the study ecoregion (Suppl. material 1). Specifically, 49% of the species are legally classified as invasive in Portugal (86 species), 21% in Spain (36 species), and 17% in France (30 species). The most represented families are Asteraceae, Poaceae, and Amaranthaceae, with 38, 25, and 13 species, respectively, whereas the rest of the families have eight or less species (20 families only have one species). Among growth forms, forb/herb is the best represented category (107 species), followed by graminoids (28), trees (14), vines (14), and shrubs (12; Table 2). Most of the invasive species are perennial (106), while annual (38) and biennial species (3) are less represented. Moreover, there are 25 species that behave as perennial or annual, and three as annual or biennial. Lastly, most species (156) are terrestrial, 10 can live in both terrestrial and aquatic ecosystems, and 9 are exclusively aquatic.

Most of the invasive species are native to the American Neotropical (76) and Nearctic (66) WWF biogeographic realms, followed by the Palearctic (41), Afrotropical (28), Indo-Malaya (22), and Australasia (12) realms (Suppl. material 1). The native range of 68 species extends over two or more realms. Regarding continent of origin, we found that most of the invasive species are native to North America (73) and South America (65). A relatively high number of species came from Africa (38) and Asia (36), whereas the native range of 16 and 12 species corresponded to Europe and Australia/Oceania, respectively. We found 60 invasive species that are native to two or more continents. Our invasive species list also includes 11 species that are native to the Mediterranean region of the Iberian Peninsula, but alien (and invasive) in our study ecoregion due to human introduction (Table 1; Suppl. material 1). Moreover, there are three hybrids that originated either from naturalized parental species in the introduced area (*Oenothera x fallax* Renner) or from artificial crosses (*Oenothera glazioviana* Micheli and *Platanus × hispanica* Mill. ex Münchh.).

Invasion status/level and population trends

Most species have low or medium invasion levels in each major area within the ecoregion (Suppl. material 1), occupying a few localized sites (low level; 35–58%) or several sites (medium level; 21–47%). Only 15–20% of species invading each major area have a high invasion level, with widespread populations (Table 2).

There are more invasive species with positive population trends (26-66%) than negative (0-10%) or neutral (0-4%) trends in each major area within the ecoregion (Suppl. material 1). However, there is a high number of invasive species with unknown population trends (26-74%).

Geographic range size

We found a high variability in the number of occurrence points at 1-km resolution that each invasive species has throughout the ecoregion (Table 2; shapefiles available at Mendeley Data repository; DOI: 10.17632/4gtnr58j2b.1). Most species (70%) have fewer than 100 registered occurrence points, 15% have 100–300 points, and 10% have 300–500 points. Only 5% of the species have 500–1,000 occurrence points (*Acacia dealbata* Link, *Prunus laurocerasus* L., *Robinia pseudoacacia, Pitto*-

Table 1. List of invasive plant species for the WWF Cantabrian Mixed Forests ecoregion. Species are separated according to their priority scores: high (2.5 to 3), medium (1.5 to 2), and low (0 to 1).

High priority species						
Acacia dealbata	Carpobrotus edulis	Hakea decurrens ¹	Paspalum dilatatum			
Acacia mearnsii	Cortaderia selloana	Lemna valdiviana	Pontederia crassipes			
Amaranthus hybridus	Crocosmia × crocosmiiflora	Ludwigia grandiflora	Reynoutria japonica			
Baccharis halimifolia	Delairea odorata	Ludwigia peploides subsp. montevidensis	Robinia pseudoacacia			
Bidens aurea	Dittrichia viscosa*	Myriophyllum heterophyllum	Sporobolus indicus			
Buddleja davidii	Elodea canadensis	Oenothera glazioviana	Tradescantia fluminensis			
Bupleurum fruticosum*	Elodea densa	Oenothera × fallax	Carpobrotus acinaciformis			
	Me	dium priority species	·			
Acacia longifolia	Cotula coronopifolia	Jacobaea maritima*	Pinus radiata			
Acacia melanoxylon	Cyperus eragrostis	Lobularia maritima*	Pittosporum tobira			
Ailanthus altissima	Eleocharis bonariensis	Lonicera japonica	Pittosporum undulatum			
Aloe maculata	Erigeron bonariensis	Matthiola incana*	Prunus laurocerasus			
Alternanthera philoxeroides	Erigeron canadensis	Muhlenbergia schreberi	Pterocarya stenoptera ²			
Amaranthus graecizans*	Erigeron floribundus	Myriophyllum aquaticum	Reynoutria sachalinensis			
Amaranthus powellii	Erigeron karvinskianus	Oenothera rosea	Reynoutria × bohemica			
Amaranthus powellii subsp. bouchonii	Erigeron sumatrensis	Oenothera stricta	Sporobolus alterniflorus			
Amaranthus retroflexus	Euphorbia polygonifolia	Opuntia elata	Sporobolus pumilus			
Arctotheca calendula	Hakea salicifolia	Oxalis latifolia	Stenotaphrum secundatum			
Artemisia verlotiorum	Hedychium gardnerianum	Oxalis pes-caprae	Symphyotrichum subulatum var. squamatum			
Arundo donax	Helianthus tuberosus	Paraserianthes lophantha	Tropaeolum majus			
Azolla filiculoides	Helianthus × laetiflorus	Paspalum distichum	Valeriana rubra*			
Bacopa monnieri	Helichrysum petiolare	Paspalum vaginatum	Xanthium strumarium			
Bidens frondosa	Hydrocotyle bonariensis	Petasites pyrenaicus	Yucca gloriosa			
Bromus catharticus	Ipomoea indica	Phytolacca americana	Zantedeschia aethiopica			
	L	ow priority species	·			
Acacia provincialis ³	Cyrtomium falcatum	Helichrysum foetidum	Potentilla indica			
Agave americana	Datura stramonium	Hydrangea macrophylla	Salpichroa origanifolia			
Ageratina adenophora	Dichondra micrantha	Impatiens balfourii	Selaginella kraussiana			
Amaranthus albus	Digitaria debilis*	Ipomoea purpurea	Senecio angulatus			
Amaranthus blitoides	Digitaria ischaemum*	Juncus tenuis	Senecio inaequidens			
Amaranthus blitum subsp. emarginatus	Disphyma crassifolium	Lepidium didymum	Senecio tamoides			
Amaranthus cruentus	Dittrichia graveolens*	Lepidium virginicum	Setaria parviflora			
Amaranthus deflexus	Dysphania ambrosioides	Matricaria discoidea	Sicyos angulatus			
Amaranthus hypochondriacus	Eleusine indica	Mesembryanthemum cordifolium	Sisyrinchium angustifolium			
Ambrosia artemisiifolia	Eleusine tristachya	Oenothera biennis	Solanum chenopodioides			
Anredera cordifolia	Eragrostis virescens	Oenothera drummondii	Solanum mauritianum			
Araujia sericifera	Erigeron primulifolius	Oxalis corniculata	Soleirolia soleirolii			
Austrocylindropuntia subulata	Eucalyptus globulus	Oxalis purpurea	Soliva sessilis			
Baccharis spicata	Euphorbia maculata	Panicum capillare	Sonchus tenerrimus*			
Bidens pilosa	Euphorbia prostrata	Panicum dichotomiflorum	Sorghum halepense			
Cenchrus clandestinus	Euphorbia serpens	Paspalum notatum	Verbena brasiliensis			
Cenchrus longisetus	Fallopia baldschuanica	Persicaria capitata	Verbena incompta			
Cenchrus setaceus	Galinsoga parviflora	Persicaria pensylvanica	Veronica persica			
Commelina communis	Galinsoga quadriradiata	Phyllostachys aurea	Vinca major			
Cotula australis	Gamochaeta coarctata	Phytolacca heterotepala	Xanthium spinosum			
Cyclospermum leptophyllum	Gladiolus undulatus	Platanus × hispanica				

*Species native to the Mediterranean region of the Iberian Peninsula, but invasive in our study ecoregion. ¹Often misidentified as *Hakea sericea* (Barker 1996). ²Often misidentified as *Pterocarya* × *rehderiana* (Muñoz-Garmendia et al. 2015) ³Often misidentified as *Acacia retinodes* (Magona et al. 2018).

Variable	Categories	Nº species (N = 175)
Growth form	Forb/Herb	107
	Graminoid	28
	Shrub	12
	Tree	14
	Vine	14
Lifespan	Annual	38
*	Biennial	3
	Perennial	106
	Annual/Biennial	3
	Annual/Perennial	25
Growing environment	Aquatic	9
0	Terrestrial	156
	Terrestrial/Aquatic	10
Continent of origin	Africa	38
0	Asia	36
	Australia	12
	Europe	16
	North America	73
	South America	65
Biogeographic realm of origin	Afrotropical	28
8 8 I	Indo-Malava	22
	Australasia	12
	Nearctic	66
	Neotropical	76
	Palearctic	41
Invasion status/level*	High	19-24
	Medium	23-63
	Low	47-63
Population trend*	Positive	31-88
1 A	Negative	0-11
	Neutral	0–6
	Unknown	35–94
Geographic range size (1×1 km cells)	~3,000	1
01 0 × ,	500-1,000	8
	100-500	44
	<100	122
Local abundance	High	60
	Medium	70
	Low	45
Environmental impacts	Massive	32
×	Moderate	59
	Limited	84
Socioeconomic impacts	Massive	18
1.	Moderate	41
	Limited	116
Type of invaded habitats	Coastal habitats	58
71	Wetlands	67
	Grasslands	13
	Shrublands	22
	Forests	30
	Vegetated man-made	128
	Constructed, industrial	40
	· · · · · · · · · · · · · · · · · · ·	

Table 2. Descriptive data (left) and invasion patterns (right) of the ecoregional invasive species pool.

*Invasion status/level and population trends were evaluated within administrative units of the ecoregion, to facilitate the assessment of the species by local experts and the application of such evaluations in local-scale management and conservation plans. *sporum tobira* (Thunb.) W.T.Aiton, *Buddleja davidii* Franch., *Acacia melanoxylon* R.Br., *Zantedeschia aethiopica* (L.) Spreng., and *Cyperus eragrostis* Lam.). The most represented species is *Cortaderia selloana* with ~3,000 occurrence points.

Species local abundance

We found that 34% of the invasive species often reach a high cover (>50%) in the invaded areas of the study ecoregion (Table 2; Suppl. material 1). Moreover, 40% of the species usually reach a cover of 5–50% (medium abundance), while only 26% of the species generally have a low local abundance (<5% of average cover).

Environmental impacts

According to the data now available, almost half of the invasive species (48%) are reported to cause limited/minimal impacts on the native biota or abiotic environment of natural and semi-natural habitats, 34% are reported to cause moderate impacts (i.e., declines in the population densities of native species, but no changes to the structure of communities or to the abiotic or biotic composition of ecosystems), and only 18% are reported to cause massive impacts (i.e., lead to the replacement and local extinction of native species, and produce changes in the structure of communities and the abiotic or biotic composition of ecosystems; Table 2; Suppl. material 1).

Socio-economic impacts

Most of the invasive species (66%) are reported to cause limited/minimal economic and social impacts on agriculture, infrastructure, landscape (visual), and human health, 24% are reported to cause moderate impacts, and only 10% are reported to cause massive impacts on the society and economy of the study ecoregion (Table 2; Suppl. material 1).

Type of invaded habitats

Vegetated man-made habitats (including crops, gardens, roadsides, hedgerows, and plantations) are the most invaded habitats, harboring populations from 73% of the invasive species (Table 2; Suppl. material 1). Coastal and wetland habitats are also invaded by a relatively high proportion of species within the ecoregion (33 and 38% of species, respectively). Constructed/industrial habitats, forests, shrublands, and grasslands have a lower proportion of the invasive species (23, 17, 13, and 7%, respectively).

Priority scores

We identified 28 invasive plant species with high priority scores, 64 species with medium priority scores, and 83 species with low priority scores (Table 1, Suppl. material 1), based on their environmental and socio-economic impacts, as well as the type of invaded habitats (natural vs. man-made).

Discussion

Invasive species pool at the ecoregion level

This paper provides the first assessment of an invasive species pool at the ecoregion scale, with comprehensive information that can be used by scientists, educators, land managers, policy makers, and other stakeholders. We selected an ecoregion that is considered a hotspot of plant invasions due to its benign climate and complex international trade networks (Gassó et al. 2012; Fernández de Castro et al. 2018), but the same framework may be applied to any ecoregion of the world (excluding ecoregions (or their parts) that include marine and connected water systems (namely rivers), which present a different invasion process; IPBES 2023). Our invasive plant list included 175 alien species present in the WWF Cantabrian Mixed Forests ecoregion, all of them producing reproductive offspring in areas distant from sites of introduction, and with potential to cause environmental alterations and economic losses (or are already causing them). We are aware that this kind of reference lists cannot be definitive since the invasion process is dynamic and can be affected by many abiotic and biotic factors (Lockwood et al. 2013; Pergl et al. 2016; Hui and Richardson 2017). Furthermore, as it is based on expert knowledge, a list such as this may not be entirely consensual, as different experts may have different knowledge and perceptions of the territory and the species present within it. Thus, we will update the published list periodically in an open repository (Mendeley Data; initial list available at DOI: 10.17632/4gtnr58j2b.1), based on future feedback from researchers and managers, or when further discussions on the assessment of individual species or regional floras suggest additions or deletions to the proposed list.

Our ecoregional list of invasive plant species contains many species included in the legal national lists developed by the countries that are part of the study ecoregion (e.g., Portuguese "Decreto-Lei n.º 92/2019", Spanish "Real Decreto 630/2013", and French "Inventaire national du patrimoine naturel"). However, we also identified species with invasive behavior within the ecoregion that are not included in existing legal national lists, because such legal lists only include invasive species that cause environmental or economic impacts. Although most of the invasive species that are not included in national lists cause limited environmental and socio-economic impacts in the ecoregion, it is important to closely monitor them and to develop management plans to prevent potential impacts in the near future.

Our ecoregional assessment of invasive plant species may help managers and policymakers to develop action plans within and across political divisions. It is important to note that in Spain, which occupies most of the study ecoregion, the power over environmental matters is transferred to autonomous regions. Thus, management actions are generally developed at spatial extents lower than the ecoregion (i.e., our ecoregion encompasses several autonomous regions). For example, managers of a given jurisdictional area (e.g., Asturias) may use information on invasive species from nearby ecologically similar areas (e.g., Galicia) to predict invasion patterns and to improve management efforts. The ecoregional assessment may also facilitate collaborations among several jurisdictional areas to prevent, early detect, and control invasive species. Thus, ecoregional evaluations allow for the optimization of management plans across ecologically homogeneous areas. Given that legislation may not be sufficient to respond to the current threats of biological invasions (Pergl et al. 2016), ecoregional assessments should be prioritized for developing management plans within individual or administrative areas of a given ecoregion.

Invasion status/level and population trends

Although we developed our framework in an ecoregion, i.e., a region with similar abiotic (e.g., climate and soil) and biotic (e.g., dominant vegetation) factors (Bailey 2004), we also accounted for administrative units within the ecoregion to assess invasion status/level and population trends. Thus, we provide information on invasion status/level and population trends for major areas that largely follow administrative boundaries within the ecoregion. We identified many species whose invasion was localized in a few sites or counties within the ecoregion (low invasion level; 35–58% of the species). Ideally, we would recommend the eradication of such species by implementing Early Detection and Rapid Response (EDRR) in administrative units of the ecoregion, which would be possible if management plans are developed shortly (Harvey and Mazzotti 2014; Robertson et al. 2020). However, in order to prevent further impacts, efforts must be focused on those species causing negative environmental and socioeconomic impacts, as we discuss in the Management priorities section below.

For the species already invading several sites (medium invasion level), eradication would be unlikely, but managers and policy makers can still contain the species and prevent further expansion across the study ecoregion (Robertson et al. 2020). However, 16–21% of species invading each major area have widespread and abundant populations (high invasion level), for which long-term management should be aimed at population suppression, resource protection, and habitats restoration (Harvey and Mazzotti 2014). We note, however, that in some situations (e.g., widespread low-impact species occupying highly disturbed areas with no conservation value), allocating a lot of resources associated with a low probability of success would not be the most optimal option, especially when it deviates resources from higher priority situations. In these cases, the "do-nothing" option should be considered, along with other measures such as burning and biological control.

Additionally, measures should be taken to prevent the introduction of species already present in some areas of the ecoregion but still absent in other(s). For example, prevention plans should be started in the western Spanish areas of the ecoregion to prevent the arrival of *Baccharis spicata* (Lam.) Baill., *Hakea decurrens* R.Br., and *Phytolacca heterotepala* H.Walter, which are already invading some areas of the Portuguese portion, and, if they enter, to early detect and quickly eradicate them. Moreover, our study indicates that we still lack information regarding population trends for many species, suggesting that field studies should focus on these species to investigate whether the invasive species is either experiencing rapid increase in distribution and abundance or, by contrast, undergoing population decrease by natural means (e.g., decline of habitat suitability due to climate change) or by human interventions (successful management).

Species occurrence and local abundance

We have built the most comprehensive and up-to-date database of invasive plant species occurrences for the ecoregion, despite some limitations typically found in biodiversity observations (e.g., most records of occurrence are only of presence, and there is no information on the absence of the species in places where we have no records). Our database contains occurrence data at 1-km resolution (or higher) that can be used by scientists, managers, and policy makers to eradicate populations and control further spread of invasive plants. It is important to note that the number of occurrence points not always reflects only the distribution of the invasive species, but also the sampling biases of the data sources. In this sense, invasive species that are easier to spot (e.g., large terrestrial plants, plants with showy flowers or other distinctive feature), more accessible, or have more awareness activities or scientific projects targeting them (e.g., *Cortaderia selloana*, *Carpobrotus* spp. N.E.Br., or *Robinia pseudoacacia*) often have more occurrence points than invasive species that are less conspicuous or more difficult to spot. Besides occurrence data, we have compiled abundance data based on expert knowledge. Species that often reach high plant cover are more difficult to eradicate (34% of the identified invasive species, e.g., *Acacia dealbata, Bidens aurea* (Aiton) Sherff, *Buddleja davidii*, and *Crocosmia* × *crocosmiiflora* (Lemoine) N.E.Br.), because control efforts significantly increase once invasive plants form dense monospecific patches. However, most species often reach low or medium local abundance, which may facilitate management plans.

Environmental and socio-economic impacts

We identified 37 invasive species with massive environmental and/or socio-economic impacts throughout the ecoregion (i.e., transformer species; sensu Richardson et al. 2000; Pyšek et al. 2004). Such species represent 21% of the invasive species present in the ecoregion and are reported to cause great impacts on community structure, ecosystem functioning, agriculture, infrastructure, landscape (visual), or human health. For example, Reynoutria japonica Houtt. and Tradescantia fluminensis Vell. form dense stands that reduce sunlight penetration and alter soil properties, suppressing native forest regeneration (Standish et al. 2001; Aguilera et al. 2010). Baccharis halimifolia L. converts the native herbaceous vegetation of coastal grasslands and estuarine communities into a landscape of monospecific woody stands, transforming the structure and function of littoral ecosystems (Fried et al. 2016; Lázaro-Lobo et al. 2022). Invasive emergent floating-leaved plants such as Ludwigia grandiflora (Michx.) Greuter & Burdet and Ludwigia peploides subsp. montevidensis (Spreng.) P.H.Raven and free-floating plants such as Pontederia crassipes Mart. and Lemna valdiviana Phil. form dense floating mats, which shade out submerged vegetation, decreasing the oxygen levels in the water column, and causing profound cascading impacts on insect assemblages and fish populations (Woodward and Quinn 2011; Lázaro-Lobo and Ervin 2021). Invasive submerged plants such as *Elodea densa* (Planch.) Casp. can outcompete other aquatic plant species and decrease species diversity (Yarrow et al. 2009). The invasive tree species Acacia dealbata and Eucalyptus globulus Labill. are fire promoters, increasing fire incidence, intensity, and spread rate, by producing and accumulating high-flammable biomass (Silva et al. 2009; Nunes et al. 2022). The pollen of Ambrosia artemisiifolia L. and Cortaderia selloana cause serious allergies and generate a second peak allergy season by blooming in late summer/fall, being a public health hazard (Nentwig et al. 2017; Rodríguez et al. 2021; Liendo et al. 2023). Lastly, control costs of invasive species can be massive. For example, containment actions on Cortaderia selloana populations in the autonomous region of Cantabria (northern Spain) were estimated to cost between €1,600,000-1,800,000 (LIFE Stop Cortaderia; http://stopcortaderia.org/).

Type of invaded habitats

As expected, vegetated man-made habitats (including crops, gardens, roadsides, hedgerows, and plantations) were more prone to be invaded than areas that experience lower human interference. Such habitats are usually subjected to periodic disturbances that generate opportunities for alien plant colonization and establishment, also acting as dispersal corridors for invasive plants (Christen and Matlack 2006; Lázaro-Lobo et al. 2020). Gardens, agricultural lands, and tree plantations are the main source of invasive plant propagules, since most invasive plants are introduced for horticulture, agriculture, and forestry purposes (Richardson 1998; Reichard and White 2001). Also, many invasive plants are often planted or introduced in seed mixtures along linear infrastructures to provide ecosystem services such as erosion control and nutrient cycling (Lázaro-Lobo and Ervin 2019). However, several species can invade other habitat types that experience low human interference, depending on their individual ecological niches. Coastal and wetland habitats are especially vulnerable to plant invasions within the ecoregion, probably because such habitats have a high resource availability (especially wetlands), and experience periodic disturbances, which make them naturally susceptible to invasion (Campos et al. 2004, 2013; Giulio et al. 2020; Lázaro-Lobo and Ervin 2021). Additionally, wetland habitats are frequently invaded by species that reproduce vegetatively (e.g., Alternanthera philoxeroides (Mart.) Griseb., Azolla filiculoides Lam., Elodea densa (Planch.) Casp., Myriophyllum heterophyllum Michx., and Pontederia crassipes), which is one of the most important predictors of invasion success (Havel et al. 2015). Forests are generally less susceptible to invasion, especially undisturbed forested areas, as found by previous research (Tomasetto et al. 2013; Iannone et al. 2016).

Management priorities

By evaluating the invasive plant species pool, we were able to identify 28 high-priority species for management at the ecoregional scale. Most such high-priority invaders (23 species) are included in legal national lists of invasive species in use by the countries that are part of the ecoregion. Some of the identified high-priority invaders already have widespread and abundant populations (i.e., high invasion level) throughout the ecoregion, such as Acacia dealbata, Carpobrotus edulis (L.) N.E.Br., Cortaderia selloana, Robinia pseudoacacia, and Tradescantia fluminensis. For those species, we propose the development of unified and cooperative control programs among the administrative units that comprise the ecoregion, aimed at population suppression, resource protection, and restoration of priority habitats. At the other extreme, some high-priority invaders have a small number of localized populations in one or a few administrative units, such as the aquatic species Elodea canadensis Michx., Myriophyllum heterophyllum, and Ludwigia grandiflora, and the terrestrial species Amaranthus hybridus L., Bupleurum fruticosum L., and Oenothera × fallax. For such invaders, we recommend the implementation of early detection and rapid response programs to eradicate the established populations (or contain them if eradication is not feasible).

It is important to note that our priority scores are based on the present situation and place great value on impacts; however, such scores will surely change in the future. For example, *Baccharis spicata* has a low priority score because nowadays it has minimal environmental impacts and invades only man-made habitats, but it has the potential to rapidly increase its expansion throughout the ecoregion, increasing its impacts on natural habitats. Thus, priority scores will also be updated in subsequent versions of the dataset.

Conclusions

This study demonstrates how the assessment of alien plant species at the ecological scale can be useful to identify priority species for management under a biogeographical basis. This information is key for optimizing resources used to control biological invasions (e.g., human effort, time, and funding), but also for evaluating long-term impacts of invasive species. We conclude that producing updated and revised catalogs of invasive species pools at the ecoregion scale is essential to evaluate biological invasions and to improve their management actions. Collecting ecoregional species pools will also be useful to prevent the local spread of invasive species from nearby areas with similar ecological and biogeographic characteristics.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: BJA, ALL. Data curation: BJA, ALL, JAC, EFP, MIRB, HM, VGG, TEDG. Funding acquisition: BJA, ALL. Investigation: BJA, JAC, VGG, HM, EFP, ALL, MIRB, TEDG. Methodology: VGG, EFP, MIRB, JAC, ALL, TEDG, HM, BJA. Resources: MIRB, VGG, EFP, TEDG, JAC, BJA, HM. Supervision: BJA. Visualization: ALL. Writing – original draft: ALL. Writing – review and editing: TEDG, JAC, EFP, BJA, MIRB, HM, ALL, VGG.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Invasive species list

Authors: Adrián Lázaro-Lobo, Juan Antonio Campos, Tomás Emilio Díaz González, Eduardo Fernández-Pascual, Víctor González-García, Hélia Marchante, María Inmaculada Romero Buján, Borja Jiménez-Alfaro

Data type: xlsx

- Explanation note: Description of the invasive plant species identified in the WWF Cantabrian Mixed Forests ecoregion.
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Supplementary material 2

Excluded species

Authors: Adrián Lázaro-Lobo, Juan Antonio Campos, Tomás Emilio Díaz González, Eduardo Fernández-Pascual, Víctor González-García, Hélia Marchante, María Inmaculada Romero Buján, Borja Jiménez-Alfaro

Data type: xlsx

Explanation note: Status of species excluded from our invasive species pool.

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Research Article

Community-level trophic characteristics and interactions between native and non-native fish: The example of the Lower Pearl River Basin of China

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Abstract

Aquatic ecosystems can harbour more than one non-native fish species and this can represent a threat due to trophic interactions with native fishes. However, research on interactions amongst multiple cooccurring native and non-native fish remains scarce. In this study, 551 organisms from 44 native fish, 11 non-native fish, 35 macroinvertebrates (of which one was non-native), together with 162 samples of basal resources were collected from six rivers of the Lower Pearl River Basin of China. Nitrogen and carbon stable isotope analysis was used to calculate community-wide trophic metrics and the degree of trophic overlap between native and non-native fish at both the community and functional feeding group level, together with diet composition. At the community level, there was a high degree of trophic niche overlap between native and non-native fish as a result of similarities in trophic characteristics. At the functional feeding group level, both native and non-native functional feeding groups demonstrated the capacity to occupy the niche space of each other. A significant trophic niche overlap, exceeding 50%, was found between non-native detritivorous and omnivorous fish, suggesting competition. The difference in diet composition between some native and non-native fish depended on the category of diet source across the rivers, suggesting dietary segregation. Albeit limited, the present findings suggest that trophic interaction between native and non-native fish is likely to reach a dynamic equilibrium status in the community owing to trophic segregation of fish species and the antagonistic effects amongst non-native fish.

Key words: Diet, impact, multiple invasions, stable isotope analysis, trophic interactions

Introduction

The management and control of non-native species has become a priority for biodiversity conservation, as invasive species are recognised as one of the major drivers of global environmental change (Pyšek et al. 2020). With the accelerating rate of globalisation, a proliferation of non-native species has been documented across an expanding array of countries and regions and this has led to numerous ecosystems facing threats from multiple invasive species (Seebens et al. 2021).



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Freshwater ecosystems are particularly vulnerable to invasions by multiple species and especially so by those fishes associated with aquaculture and the aquarium trade (Bernery et al. 2022). However, research has mainly focused on individual non-native fish species, overlooking the intricate interactions amongst multiple co-occurring native and non-native fish (Coughlan et al. 2022). Given that interactions amongst these species can trigger cascading effects throughout the entire food web, gaining a deeper understanding is crucial for devising ecosystem-level management strategies to address multiple species invasions (e.g. Rogosch and Olden (2020)).

The co-existence of species within communities, driven by species interactions, predominantly stems from niche differences (e.g. disparities in environmental requirements), facilitating resource partitioning and reduced interspecific competition (Giam and Olden 2016; Buche et al. 2022). Differences in the niches occupied by native and non-native species can profoundly influence the outcomes of biological invasions (Li et al. 2019). Therefore, a fundamental inquiry regarding the consequences of invasions by multiple non-native fish revolves around whether the trophic niche of co-occurring non-native fishes differs from that of their sympatric native counterparts. Assessing the extent of niche overlap between sympatric native and non-native fish serves as a valuable metric for evaluating the intensity of competition (Tran et al. 2015; Haubrock et al. 2021). In this respect, stable isotope analysis emerges as a powerful analytical tool for elucidating such interactions across various trophic levels (McCue et al. 2020; Balzani and Haubrock 2022).

The Lower Pearl River Basin of China faces a heightened risk of non-native fish invasions due to intensive aquaculture and the ornamental fish trade (Wei et al. 2019). A recent investigation revealed that 14 non-native fish species have established self-sustaining populations in the area (Wei et al. 2019). Amongst these species, Mrigal carp Cirrhinus mrigala, redbelly tilapia Coptodon zillii, Nile tilapia Oreochromis niloticus and armoured catfish Pterygoplichthys spp., which are omnivores and detritivores, have established large self-sustaining populations and co-occurred in the Pearl River Basin (Gu et al. 2020). These sympatric species have similar diets, primarily constituting of detritus, dead organisms, macroinvertebrates and aquatic plants and, accordingly, compete for food (Froese and Pauly 2023). At the same time, competitive biotic resistance, which is driven by competition amongst species exploiting the same resources, tends to be weaker in freshwater communities compared to marine and terrestrial communities due to lower species diversity and availability of unexploited niches (Moyle and Light 1996; Shurin et al. 2006; Alofs and Jackson 2014). These characteristics can accelerate the invasion of non-native species in freshwater communities (Moyle and Light 1996). On the other hand, native fish in freshwater ecosystems can be susceptible to competition with non-native fish due to the latter's advantage in terms of life-history traits (Bohn et al. 2008; Rehage et al. 2020). Studies have suggested that non-native fish are characterised by omnivory, faster growth, higher fecundity, earlier maturity, higher ability to colonise various habitats, higher tolerance to stress and higher phenotypic plasticity relative to native fish (Cucherousset et al. 2009; Liu et al. 2017). Overall, the ecological dynamics of competitive native and multiple non-native species are inherently complex, involving the interaction between native and non-native fish, as well as amongst non-native fish, making the consequences of invasions by multiple fish difficult to predict.

This study employed nitrogen (δ^{15} N) and carbon (δ^{13} C) stable isotope analysis to achieve three objectives: (i) elucidate differences in trophic characteristics between native and non-native fish at the community level; (ii) quantify the degree of niche overlap between native and non-native fish at the community and functional feeding group levels; and (iii) determine whether native and non-native fish have similar diet composition. The outcomes of this study are expected to provide a deeper understanding of the interactions between multiple native and non-native fish, which is crucial for identifying the consequences of multiple species invasions.

Methodology

Sampling and processing

Sampling in the Lower Pearl River Basin included the rivers Beijiang, Dongjiang, Liuxihe, Xijiang, Xizhijiang and Zengjiang (Fig. 1). The region is characterised by a subtropical monsoon climate, with an annual mean temperature of 21.8 °C and mean annual rainfall of 1790 mm, primarily occurring between April and September (Li et al. 2013). In total, 551 organisms from 44 native fish species, 11 non-native fish species and 35 macroinvertebrate species of which one was non-native (Suppl. material 1: table S1) were collected together with 162 samples of basal resources from the six rivers during summer and early autumn in 2020. Only adult fish were sampled to reduce the effects of ontogeny. For the organisms, one to three individuals were sampled per species per site; for the basal resources, three samples were collected per site. Sampling at such spatial extent ensured the ability to generalise the findings from this study (e.g. Farly et al. (2019); Reis et al. (2020); Filazzola and Cahill (2021)). Fish were captured using six gillnets (mesh size 45 mm, length 5 m) and six shrimp traps (mesh size 4 mm, length 5 m), which were randomly placed at the sampling sites for ≈ 12 h. The sampled fish were moved to a portable refrigerator (-20 °C) and shipped to the laboratory. Fish were identified to species level and categorised into their predominant functional feeding group (FFG) (after Wang et al. (2019): Suppl. material 1: table S1). Standard length (SL) and body weight were measured for each specimen. A sample of dorsal muscle tissue was collected from each fish for further laboratory processing. The use of animals in this study complied with the National Research Council's Guide for the Care and Use of Laboratory Animals.

To predict the diet composition of native and non-native fish, potential food sources were collected from the six rivers where fish were sampled. Aquatic insects were captured using a D-shaped net (edge length of 30 cm and pore size of 500 µm: Rosati et al. (2016)). Snails and bivalves were sampled by hand and their soft tissues collected for isotope analysis. All macroinvertebrate specimens were identified to the lowest practical taxonomic level, typically the species and their body length and weight were measured. Macroinvertebrate taxa were categorised, based on their predominant FFG (after Tachet et al. (2002): Suppl. material 1: table S1). Riparian plants accessible to fish were also collected and identified to the species level. Plant species were classified as C3 and C4, based on their δ^{13} C value (i.e. C3 plants: -36‰ to -20‰; C4 plants: -15‰ to -7‰: Farquhar et al. (1989)). For fine particulate organic matter (FPOM), the surface layer of the sediment was agitated to suspend particles in the water and a sample of the suspension was collected in an acid-washed plastic bottle. Coarse particulate organic matter (CPOM) was manually collected. Periphyton was removed from five to six rocks using a toothbrush and the suspension was preserved in an acid-washed plastic bottle. Seston, primarily consisting of phytoplankton and detritus, was collected using a seston sampler (mesh size: 0.064 mm) and the slurry transferred to a plastic bottle that had been washed with acid. All samples were promptly preserved at -20 °C.



Figure 1. Map of the six rivers of the Lower Pearl River Basin of China sampled for native and non-native fish, macroinvertebrates and basal resources.

Stable isotope analysis

All samples were dried at 60 °C for 48 h and then ground to powder using a mortar and pestle. For small-bodied macroinvertebrates, two to three individuals were homogenised into one sample. Basal resources including CPOM, FPOM, periphyton, plants and seston were also dried, ground and acidified to remove inorganic carbon. The powdered samples were loaded into individual tin capsules and weighed. Up to three replicates of each animal species and basal resources per river were combusted. The N and C content and isotope ratios were determined using a continuous-flow carrier-gas system (Conflo) equipped with a stable isotope mass spectrometer (Delta V Advantage, Thermo Finnigan, Germany) and an elemental analyser (Thermo Fisher, USA) at Wuhan Botanical Garden, Chinese Academy of Sciences. Replicates of isotopic standard samples (urea) were processed to calibrate for any potential drift (Jackson et al. 2020). The $\delta^{15}N_{AIR}$ and $\delta^{13}C_{V-PDB}$ of urea were -0.30% and -42.63%, respectively, with an uncertainty value of 0.2% (95% confidence level). Precisions, calculated as (SD/mean) *100, for the repeated measurements of urea, were 0.8% (n = 23) for $\delta^{15}N$ and 0.3% (n = 23) for $\delta^{13}C$.

The $\delta^{15}N$ and $\delta^{13}C$ were corrected to compare differences amongst rivers. Accordingly, $\delta^{15}N$ was converted to trophic position (TP) as per Olsson et al. (2009):

$$TP = 2 + (\delta^{15}N_{fish} - \delta^{15}N_{prev}) / 3.4,$$

where $\delta^{15}N_{fish}$ is the nitrogen stable isotope ratio of each individual fish and $\delta^{15}N_{prey}$ is the average $\delta^{15}N$ value of the macroinvertebrate prey resources from each river. In this study, the bivalves of the primary consumers and *Angulyagra polyzonata* were used in the calculation. Bivalves and *A. polyzonata*, which were widely distributed across the six rivers, are long-lived filter-feeders and have relatively simple diet sources. Note that the macroinvertebrates were enriched in $\delta^{15}N$ due

to the pollutions from domestic sewage in the Lower Pearl River (e.g. Bode et al. (2014); Xue et al. (2023)). The constant 2 is the empirical value of the trophic position of the primary consumers and the constant 3.4 is the fractionation factor between adjacent trophic levels (Post 2002). The δ^{13} C was corrected as follows:

$$\delta^{13}C_{\text{corr}} = (\delta^{13}C_{\text{fish}} - \delta^{13}C_{\text{meanMI}}) / CR_{\text{MI}}$$

where $\delta^{13}C_{corr}$ is the corrected $\delta^{13}C$, $\delta^{13}C_{fish}$ is the $\delta^{13}C$ of each individual fish from each river, $\delta^{13}C_{meanMI}$ is the average $\delta^{13}C$ of the bivalves and *A. polyzonata* from each river and CR_{MI} is the $\delta^{13}C$ range ($\delta^{13}C_{max}\delta^{13}C_{min}$) of the bivalves and *A. polyzonata* (Olsson et al. 2009).

Statistical analysis

Trophic characteristics

The effect of fish origin (Origin: native, non-native) and FFG on $\delta^{13}C_{corr}$ and TP was analysed by linear mixed modelling using the R package lme4. In the models, $\delta^{13}C_{corr}$ and TP were the response variables, Origin and FFG the fixed effects, River and SL the random effects, as follows:

$$\delta^{13}C_{corr} \sim Origin + FFG + Origin:FFG + 1 / River + 1 / SL$$

TP ~ Origin + FFG + Origin:FFG + 1 / River + 1 / SL

Layman metrics were computed for the native and non-native fish in each river using the R package SIBER (Layman et al. 2007). Trophic structure was described by six community-wide Layman metrics for the δ^{15} N and δ^{13} C ranges, total area (TA), mean distance to centroid (CD), standard deviation of nearest neighbour distance (SDNND) and mean nearest neighbour distance (MNND). The influence of fish origin on the Layman metrics was tested by permutational (univariate) analysis of variance, based on a one-way design using the adonis function of the vegan R package. A Euclidean distance matrix was used, with 1000 permutations of the raw data and statistical effects evaluated at $\alpha = 0.05$.

Niche overlap

The probability of overlap between the isospace of native and non-native fish was estimated by Bayesian analysis using the R package nicheROVER, which is not sensitive to sample size, with a normal-independent-inverse-Wishart prior to simulate the posterior distribution of the models' parameters (Swanson et al. 2015). The overlap metric is directional and represents the probability that an individual from Group A will be found in the niche of an individual from Group B. Niche area is defined as the region in the δ^{15} N and δ^{13} C bivariate space with 95% probability, using a Bayesian approach to account for uncertainty (Swanson et al. 2015). Mean overlap of native and non-native fish was calculated using Bayesian 95% credible intervals based on 10,000 iterations (Swanson et al. 2015). Fish niche overlap was estimated at community level and FFG level using this model. At the community level, native and non-native fish were pooled respectively for each river. The analysis was also conducted at FFG level

to investigate the interaction amongst non-native FFGs, as well as between native and non-native FFGs. Each FFG was then separated for native and non-native groups for each river. Samples with more than five individuals were included in the models. The analysis was conducted separately for each river to account for spatial variation in isotopic baselines. The probability of niche overlap between two groups was considered to be biologically significant when > 50%. Raw isotopic data were used in the analysis. In this study, a Bayesian approach was preferred over a classical (frequentist) approach owing to the small sample sizes resulting from the sampling design that accounted for the diversity of watercourses in the Lower Pearl River Basin and the community-wide extent of the analyses. The use of credible intervals (analogous to confidence intervals in frequentist statistics) was, therefore, deemed a more informative option than setting significance levels (Hilborn and Mangel 1997).

Diet composition

The relative proportions of potential sources contributing to the diet of native and non-native fish in each river were analysed using a Bayesian mixing model with R package MixSIAR (Stock et al. 2018). Estimates were based on the mean \pm SD δ^{15} N and δ^{13} C of the basal resources and with Species as the random factor. Only fish species with more than three samples were selected for analysis. To account for spatial variation in the isotopic ratios of diets, food resources and fish were analysed separately for each river. Resources were pooled into six groups (i.e. C3 plants, C4 plants, CPOM/FPOM, macroinvertebrates, periphyton, seston) for each site, based on the overlap in isotopic space for the individual sources and the category of the resources (Suppl. material 1: fig. S1). All mixed models were conducted using lipid-corrected δ^{13} C values, which were adjusted, based on equations provided for macroinvertebrates (Post et al. 2007) and fish (Kiljunen et al. 2006). These adjustments were made for those cases where the C/N ratios were \geq 3.5. Trophic discrimination factors (TDFs) of 2.38 ± 0.37‰ for δ^{15} N and 0.96 ± 0.26 ‰ for δ^{13} C were used to adjust for variation in isotopic discrimination between fish and their food sources (Wang et al. 2021). Simulation of Bayesian mixing polygons was performed for all consumers by Markov Chain Monte Carlo with 1,500 iterations to ensure all individuals fell within a 95% mixing polygon (Smith et al. 2013). The model used the Process * Residual error term for the consumer, with Process for the variation of consumer and Residual for the variation of sampling process and consumer specialisation (Stock and Semmens 2016). Uninformative priors (all values equally between 0 and 1) were used for the models. These were run using a Markov Chain Monte Carlo simulation with 50,000 to 300,000 iterations until model convergence was reached, as assessed by Gelman-Rubin and Geweke diagnostic tests (Stock and Semmens 2016). The precision of the estimates was evaluated by examining the spread of the posterior distribution (Stock and Semmens 2016).

Permutational multivariate analysis of variance was conducted with medium values using the adonis function from the vegan R package to investigate the impacts of River, FFG, Origin and their interaction terms on the potential diet contribution to the fish. A Bray-Curtis dissimilarity measure was employed, with 9999 unrestricted permutations of the raw data and with statistical effects evaluated at $\alpha = 0.05$. Differences in diet composition amongst fish species were determined by their 95% credible intervals, with overlapping credible intervals indicating no differences amongst fish species (Stock et al. 2018). All analyses in this study were performed using the R language (R x64 4.1.2).

Results

Trophic characteristics

The TP and $\delta^{13}C_{corr}$ of native fish were higher, though not statistically different, than that of non-native fish (Fig. 2A, B). The TPs of invertivores and piscivores were higher than those of the other FFGs (Table 1, Fig. 2C). The TP of native piscivores was higher than that of non-native piscivores (Suppl. material 1: fig. S2). The $\delta^{13}C_{corr}$ of herbivores and omnivores were higher than that of the other FFGs (Table 1, Fig. 2D).

Layman metrics did not differ between native and non-native fish for the δ^{15} N and δ^{13} C ranges, nor for TA and CD (respectively: *F* = 2.25, *p* = 0.33; *F* = 3.53, *p* = 0.17; *F* = 55.57, *p* = 0.18; *F* = 0.39, *p* = 0.58). However, the δ^{15} N and δ^{13} C ranges of native fish were wider than those of non-native fish (Fig. 3A–D). Conversely, SDNND and MNND differed (respectively: *F* = -0.73, *p* = 0.04; *F* = -0.53, *p* = 0.005), with the native fish having lower values than non-native fish (Fig. 3E, F).

Niche overlap

At the community level, the probability that a non-native fish would be found in the niche of a native fish was higher compared to the opposite (Fig. 4, Suppl. material 1: fig. S3). River-wise, this probability was highest in Beijiang (94.6%), followed by Xizhijiang (91.2%), Dongjiang (91.1%), Zengjiang (89.2%), Liuxihe (86.3%) and Xijiang (76.97%). The probability that native fish would be found in the niche of a non-native fish was highest in Xijiang (90.4%), followed by Xizhijiang (83.0%), Liuxihe (81.0%), Beijiang (78.1%), Zengjiang (75.9%) and Dongjiang (43.8%).

At the FFG level, the niche space of non-native detritivores and omnivores significantly overlapped with that of the other native FFGs (Fig. 5A, B). In Beijiang, non-native detritivores occupied 78.93% of the niche space of native planktivores, 90.85% of native piscivores, 92.86% of native invertivores and 97.46% of native omnivores. Non-native omnivores occupied 71.48% of the niche space of native piscivores, 72.92% of native planktivores, 74.30% of native invertivores and 88.27% of native omnivores. On the other hand, native omnivores, invertivores and piscivores occupied 64.80%, 73.75% and 81.93% of the niche space of non-native detritivores, respectively. Native piscivores occupied 58.24% of the niche space of non-native omnivores. Non-native omnivores occupied 59.60% of the niche space of non-native detritivores, while non-native detritivores occupied 55.64% of the niche space with non-native omnivores. The niche sizes of these non-native fish were similar, except for the niche size of native planktivores which was smaller than that of native omnivores (Fig. 5C, Suppl. material 1: table S2).

In Dongjiang, non-native detritivores occupied 67.73% of the niche space of native omnivores, while native detritivores and planktivores occupied 51.00% and 72.76% of non-native detritivores. The niche sizes of these non-native fish were similar, except for the niche size of native detritivores which was smaller than that of native omnivores (Fig. 5D, Suppl. material 1: table S2). In Liuxihe, non-native detritivores occupied 62.74% of the niche space of planktivores and 72.57% of native omnivores. On the other hand, native planktivores, omnivores, invertivores and piscivores occupied 76.88%, 77.98%, 83.58% and 91.79% of the niche space of non-native fish were similar, except for the niche size of native invertivores which was smaller than that of non-native detritivores (Fig. 5E, Suppl. material 1: table S2).



Figure 2. Differences between native and non-native fish in trophic position (TP) and corrected δ^{13} C and amongst functional feeding groups (FFG). Significant effects are indicated as *** *p* < 0.001, ** *p* < 0.01 and † *p* < 0.1.

Table 1. Fixed and random effect coefficients for a linear mixed model describing the effect of Origin (native, non-native), functional feeding group (FFG) and their interaction on trophic position (TP) and corrected δ^{13} C (δ^{13} C_{corr}) for fish in six rivers of the Lower Pearl River Basin of China. SL = standard length. See also Fig. S2. Significant results indicated as *** (p < 0.001), ** (p < 0.01), * (p < 0.05) and † (p < 0.1).

Effect	Source	ТР	$\delta^{13}C_{corr}$
Random	SL	0.11	0.03
	River	0.79	0.82
Fixed	Intercept	1.64	0.76
	Origin (Native)	0.61	0.56
	FFG (Herbivore)	-0.33	3.24**
	FFG (Invertivore)	3.41***	-0.36
	FFG (Omnivore)	1.52	2.10*
	FFG (Piscivore)	-1.67†	1.10
	FFG (Planktivore)	0.03	-1.84†
	Origin (Native) * FFG (Omnivore)	-0.87	-0.22
	Origin (Native) * FFG (Piscivore)	2.34*	-0.43



Figure 3. Layman metrics for native and non-native fish for: (A) δ^{15} N range, (B) δ^{13} C range, (C) total area (TA), (D) mean distance to centroid (CD), (E) standard deviation of nearest neighbour distance (SDNND), (F) mean nearest neighbour distance (MNND). Insets show the Layman metrics pooled across the six rivers. Significant effects at $p \le 0.05$ are marked with an asterisk, at $p \le 0.01$ marked with two asterisks.

In Xijiang, non-native detritivores occupied 72.57% of the niche space of native omnivores. On the other hand, native planktivores, piscivores and omnivores occupied 75.30% 85.98% and 88.94% of the niche space of non-native detritivores, respectively, while native planktivores, omnivores and piscivores occupied 59.74%, 69.94% and 82.82% of non-native omnivores, respectively. Non-native detritivores occupied 74.35% of the niche space of non-native omnivores, while non-native omnivores occupied 66.31% of non-native detritivores. The niche sizes of these non-native fish were similar, except for the niche size of native planktivores which



Figure 4. Bi-dimensional projections (with 95% CI) of the $\delta^{15}N$ and $\delta^{13}C$ isotopic niche region for native (red) and non-native (blue) fish.

was smaller than that of non-native detritivores and native omnivores (Fig. 5F, Suppl. material 1: table S2). In Xizhijiang, non-native detritivores occupied 88.15% of niche space of native omnivores, whereas native omnivores occupied 59.61% of non-native detritivores. The niche size of native omnivores was similar with that of non-native detritivores (Fig. 5G, Suppl. material 1: table S2). In Zengjiang, non-native detritivores occupied 98.75% of the niche space of native omnivores. On the other hand, native planktivores occupied 96.34% of the niche space of non-native detritivores. The niche sizes of these non-native fish were similar, except for the niche size of native omnivores which was broader than non-native detritivores and native invertivores (Suppl. material 1: table S2, Fig. 5H).



Figure 5. Niche overlap estimates and niche size of native and non-native functional feeding groups (FFG). Only estimates > 50% are shown. (A) Probability of non-native fish FFGs (light red) overlapping with native fish FFGs (light blue) indicated by the direction of the arrows. Line thickness indicates the degree of niche overlap with rivers labelled above the lines. (B) Probability of native fish FFGs overlapping with non-native fish FFGs. (C-H) Niche size of overlapping functional feeding groups in the six rivers. The differences in niche sizes among FFGs can be found in Table S2. Fish FFG abbreviations: Detr_nat = detritivorous native; Detr_nonnat = detritivorous non-native; Herb_nat = herbivorous native; Inve_nat = invertivorous native; Omni_nat = omnivorous native; Omni_nonnat = omnivorous non-native; Pisc_nat = piscivorous native; Plan_nat = planktivorous native. River abbreviations: BJ = Beijiang; DJ = Dongjiang; LXH = Liuxihe; XJ = Xijiang; XZJ = Xizhijiang; ZJ = Zengjiang.

Diet composition

River significantly affected the potential diet composition of fish, but not the interactions with Origin and FFG (Table 2). The difference in diet composition between some native and non-native fish depended on the category of diet source across the rivers (Fig. 6, Suppl. material 1: tables S3–S8). In Beijiang, the potential food sources contributed equally to the diet of native and non-native fish, except for macroinvertebrates (Fig. 6A, Suppl. material 1: table S3). *Pterygoplichthys* spp. had a higher probability to consume macroinvertebrates than native omnivores *Acheilognathus macropterus*, *Osteochilus salsburyi*, large-scale loach *Paramisgurnus dabryanus*, the non-native omnivore streaked prochiloid *Prochilodus lineatus*, the invertivore *Hemibarbus umbrifer*, as well as Hong Kong catfish *Clarias fuscus* and

Source	df	R^2	F	p
River	5	0.330	8.88	< 0.01
FFG	5	0.050	1.42	0.12
Origin	1	0.001	0.20	0.93
River*FFG	18	0.110	0.86	0.77
River*Origin	5	0.050	1.37	0.14
FFG*Origin	2	0.010	0.87	0.53
River*FFG*Origin	3	0.004	0.18	1.00
Residual	91	0.390		

Table 2. Permutational multivariate analysis of variance for the effect of River, functional feeding group (FFG), Origin (native, non-native) and their interaction terms on the diet composition (i.e. median diet proportions) of fish. Significant effects ($\alpha = 0.05$) in bold.

Amur catfish Silurus asotus. In Dongjiang, the probabilities of all potential food sources contributed equally to the diet of native and non-native fish, except for macroinvertebrates (Fig. 6B, Suppl. material 1: table S4). The non-native detritivores C. mrigala, O. niloticus and Pterygoplichthys spp. had a lower probability to consume macroinvertebrates relative to the native omnivore Megalobrama terminalis. In Liuxihe, the probabilities of all potential food sources contributed equally to the diet of native and non-native fish, except for C4 plant. Native omnivore P. dabryanus had a higher probability to consume C4 plants than non-native detritivores C. mrigala and Pterygoplichthys spp (Fig. 6C, Suppl. material 1: table S5). In Xijiang, no difference was found in the probability of diet contribution between native and non-native fish (Fig. 6D, Suppl. material 1: table S6). In Xizhijiang, the potential diet contributed in similar probabilities to that of native and non-native fish, except for CPOM/FPOM (Fig. 6E, Suppl. material 1: table S7). The non-native detritivore C. mrigala had a higher probability to consume CPOM/FPOM than the native omnivores A. macropterus and Carassioides acuminatus, the planktivore silver carp Hypophthalmichthys molitrix, the invertivore zig-zag eel Mastacembelus armatus and the non-native omnivore C. zillii and detritivore Pterygoplichthys spp. Native detritivore Cirrhinus molitorella had a higher probability to consume CPOM/FPOM than non-native C. zillii and Pterygoplichthys spp. In Zengjiang, the potential food source contributed in similar probabilities to the diet between native and non-native fish, with the exception of C3 plants and CPOM/FPOM (Fig. 6F, Suppl. material 1: table S8). The non-native piscivore African sharptooth catfish Clarias gariepinus had less probability to consume C3 than the native piscivores Hemibagrus macropterus, yellow catfish Tachysurus fulvidraco, the invertivores Eleotris oxycephala, M. armatus and Rhinogobius giurinus, while it had a higher probability to consume CPOM/FPOM than these piscivores and invertivores.

Discussion

Although previous studies have indicated that the invasion of non-native fish may lead to a reduction in the trophic position of native fish species as a result of interspecific competition (Britton et al. 2018), the current study observed that, despite no significant differences, these non-native fish tended to occupy a lower trophic position relative to the native fish. In the Lower Pearl River Basin, non-native fish species, associated with aquaculture and aquarium trade, are primarily detriviores



Figure 6. Comparison of the probability of diet contribution between native (bold) aquatic organisms and non-native (italics) fish. Species name abbreviations in Suppl. material 1: table S1.

and omnivores, contrasting with other study areas where non-native fish are predominantly piscivorous and associated with recreational fishing activities (Eby et al. 2006; Cucherousset et al. 2012). The occupancy of lower trophic positions by non-native fish may confer an advantage, as they can exploit more readily available basal resources such as detritus, plants and algae within the recipient ecosystem (Moyle and Light 1996; Gido and Franssen 2007; Liew et al. 2016). These basal resources typically exhibit lower δ^{15} N than animal prey, which consequently leads to fish that consume these resources having lower δ^{15} N, thereby occupying a lower trophic position (Post 2002). In this regard, non-native piscivore (i.e. *C. gariepinus*) occupied a significantly lower trophic position than native piscivores, which is consistent with its diet preference on δ^{15} N depletion diets in the invaded rivers (e.g. Low et al. 2022). Utilisation of basal resources could have contributed to the observed trophic overlap in this study between native and non-native fish, as these resources are also essential for the native fish.

No discernible differences in trophic structure and diet composition were observed between native and non-native fish, leading to a high degree of overlap. These findings suggest that both native and non-native fish may face intense competition. Although some native fish exhibited distinct dietary composition from non-native fish across the study rivers, native fish species may confront heightened competition not only from non-native fish, but also amongst themselves. This is attributable to the increased trophic redundancy within the native fish community, with native fish depending on a limited subset of resources (Layman et al. 2012). Thus, native fish are anticipated to experience exacerbated adverse effects from interspecific competition.

Interspecific interactions between native and non-native fish have been shown to result mainly in negative or neutral impacts (Bohn et al. 2008; Jackson et al. 2016). The exclusion of native fish due to competition with non-native fish may not be immediately apparent when food resources are readily available to both native and non-native fish (Britton et al. 2019). In this study, at the community level, non-native fish had a higher probability to occupy the niche space of native fish than the opposite scenario. This result suggests that the ecological niche space of native fish may be reduced. Studies have demonstrated that fish invasions have resulted in dietary changes and trophic niche reduction of native fish (e.g. Parvez et al. (2023); Quintana et al. (2023)). Nonetheless, not all non-native FFGs had a high likelihood of encroaching upon the niche space of native FFGs in this study. This observation suggests that extrapolating community-level interaction outcomes from interactions at the FFG level can result in estimation errors. This is because the stable isotope spaces of communities are made of points clustered by species. Additionally, the species in two communities could be separated by empty space. Thus, the stable isotope spaces of two communities may overlap without any actual species overlap. Consequently, studies should focus on community-level interactions to gauge more accurately the collective impacts of multiple fish invasions.

The use of FFG level analysis clarifies interactions between native and non-native fish in the Lower Pearl River Basin. Non-native detritivorous fish had a high probability to occupy the niche space of native FFGs. The non-native detritivorous fish *C. mrigala*, *O. niloticus* and *Pterygoplichthys* spp. are the most abundant non-native fish species in the Pearl River Basin (Gu et al. 2019). Despite detritus representing the primary component of their diet, these species demonstrate a high degree of dietary plasticity, which allows them to incorporate a diverse array of food sources into their diet. For example, as a detritivorous fish, *Pterygoplichthys* spp. were anticipated to feed primarily on detritus and algae (Froese and Pauly 2023). However, based on gut content analysis, *Pterygoplichthys* spp. have been found to consume also benthos, plants and carcasses (Reinas et al. 2022). The results of stable isotope analysis in this study revealed that macroinvertebrates made a relatively high contribution to the diet of *Pterygoplichthys* spp. in Beijiang, where richness and abundance of macroinvertebrates are high. While *O. niloticus* may not directly impact piscivorous fishes and major carps, its trophic niche has been found to overlap with that of native fishes due to its adaptable feeding habits (Khan and Panikkar 2009; Henson et al. 2018). *Cirrhinus mrigala* also exhibits a broad dietary spectrum, with its primary food sources encompassing a variety of plant matter, invertebrates, algae and zooplankton (Zhang et al. 2024). All of this could explain why non-native detritivorous fish can colonise the trophic niche of a diverse array of native fish in different FFGs.

The niche space of native omnivorous fish was relatively broader than that of non-native detritivorous fish in Zengjiang, which might mitigate the competitive pressure exerted by non-native fish. Native omnivorous, piscivorous and planktivorous fish also had high probabilities of occupying the niche space of non-native detritivorous and omnivorous fish. These results suggest that non-native fish might also face intense competition from native fish. Overall, this study has revealed that non-native fish have established novel trophic interactions with native fish within the community. However, observations also indicated that some native FFGs demonstrated minimal interactions with non-native FFGs, suggesting variability in the degree of ecological integration (see Suppl. material 1: fig. S3). This variability depends on factors such as quantity and quality of food resources within the habitat, degree of environmental disturbance as well as foraging strategies and population density (Liew et al. 2016; Wang et al. 2019; Almela et al. 2021; De Santis et al. 2021). Fish species, especially those occupying specialised trophic niches, may be more vulnerable to the adverse effects of interactions with non-native fish (Almela et al. 2021). Conversely, species with a wider dietary breadth generally exhibit greater resilience against competitive pressures by non-native species (Collier et al. 2018). However, native fish species whose trophic niche sizes are similar to those of non-native fish may face direct competition in their diet (Harris et al. 2022). These findings suggest that novel interactions between native and non-native fish can lead to a dynamic equilibrium within the community rather than causing its collapse (e.g. Alofs and Jackson (2014)).

Co-occurring non-native fish species have the potential to alter community structure through direct or indirect interactions, either facilitating or suppressing one or both invaders (Coughlan et al. 2022; Crone et al. 2023). A meta-analysis has shown that the majority of interactions amongst invaders are neutral (Jackson 2015). This study has revealed a significant trophic niche overlap, exceeding 50%, between non-native detritivorous and omnivorous fish. This finding suggests the potential for competition amongst these non-native fish, as they have similar dietary and niche size.

Conclusions

Understanding the consequence of interactions between multiple non-native fish and native aquatic organisms is a major challenge to manage multiple invasions (e.g. Guareschi et al. (2021)). The results of this study have suggested that native and non-native fish may experience intense competition from each other due to their similar trophic characteristics. Some native fish might alleviate the impacts from non-native fish due to their larger niche size. Some native and non-native fish have different types of foraging behaviour and can use distinct food resources to avoid direct competition (e.g. Zaia Alves et al. (2020)). In this study, the trophic niche of non-native detritivorous fish was significantly overlapped with distinct FFGs, such as invertivores, piscivores and planktivores, which have different trophic traits and foraging behaviour (Froese and Pauly 2023). The interspecific competition amongst non-native fish might also alleviate the negative impacts of non-native fish on native fish (e.g. Van Zwol et al. (2012); Liu et al. (2018)). Albeit limited, the present findings suggest that trophic interaction between native and non-native fish is likely to reach a dynamic equilibrium status in the community owing to the trophic segregation of fish species and antagonistic effect amongst non-native fish.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

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Data availability

The datasets and R code generated in this study are available from the first author upon request.

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Supplementary material 1

Additional information

Authors: Hui Wei, Lorenzo Vilizzi, Mingsi Zhang, Ying Jiang, Meng Xu, Miao Fang, Fandong Yu, Lu Shu, Xuejie Wang, Dangen Gu

Data type: docx

- Explanation note: This file includes three supplementary figures and eight tables. The titles of the figures and tables are listed as following: **fig. S1.** Biplot of δ^{15} N vs δ^{13} C for food resources. **fig. S2.** Differences in trophic position (left) and corrected δ^{13} C‰ (right) between native and non-native detritivore, omnivore and piscivore fish. **fig. S3.** Niche overlap estimates of native and non-native functional feeding groups in six rivers of the Lower Pearl River Basin of China. **table S1.** Fish and macroinvertebrates, with indication of feeding group and origin, sampled from the Lower Pearl River Basin of China. **table S2.** The mean, standard error and 95% credible intervals of the niche size for native and non-native functional feeding group in six rivers. **tables S3–S8.** MixSIAR results for the probability of diet contribution for native and non-native fish in Beijiang, Dongjiang, Liuxihe, Xijiang, Xizhijiang and Zengjiang. Species name abbreviations in table S1.
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Research Article

Evaluating spatially explicit management alternatives for an invasive species in a riverine network

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Abstract

Invasive species have substantial ecological and economic costs and removing them can require large investments by management agencies. Optimal spatial allocation of removal effort is critical for efficient and effective management of invasive species. Using a series of ecologically informed model simulations, we evaluated and compared different spatially explicit removal strategies for invasive rusty crayfish (Faxonius rusticus) in the John Day River, USA. We assessed strategies in terms of their performance on three likely management objectives: suppression (minimise overall population abundance), containment (minimise the spatial extent of invasion) and prevention (minimise spread into a specific area). We developed five spatial removal strategies to achieve those objectives, denoted as: Target Abundance (removal at locations with the highest population abundance), Target Growth (removal at locations with the highest population growth), Target Edges (removal at the most distant locations in the river), Target Downstream (removal at the most downstream invaded segments on the Mainstem), and Target Random (removal at randomly selected locations). Each strategy was assessed at various effort levels, referring to the number of spatial segments in the river in which removals were conducted, after seven years of management. We identified the alternative that best achieved each objective, based on decision criteria for risk-neutral and risk-averse decision-makers and further evaluated strategies based on Pareto efficiency, which identifies the set of alternatives for which an improvement on one objective cannot be had without a decline in performance on another. We found that Target Abundance and Target Growth strategies best achieved the suppression objective, for risk neutral and risk averse decision-makers, respectively and Target Downstream was always best in achieving the prevention objective across both types of decision-makers. No single strategy consistently performed best in terms of the containment objective. In terms of all three objectives, Target Downstream was consistently Pareto efficient across all levels of management effort and both decision criteria. The modelling framework we provided is adaptable to a variety of riverine invasive species to help assess and compare spatial management strategies.

Key words: Aquatic invasive species, crayfish, invasive species management, spatially explicit model



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Introduction

Invasive species are a primary threat to global biodiversity, economies and human health (Pyšek and Richardson 2010; Early et al. 2016; Diagne et al. 2021). Managers of invasive species often seek to reduce two of the main contributors to negative species impacts: population abundance and range extent (Parker et al. 1999; Kumschick et al. 2015). However, desired management outcomes can be challenging to achieve due to imperfect detection, the ineffectiveness of available management actions and invasive species whose rapid growth and spread can largely nullify the effect of management (Rendall et al. 2021; Cuthbert et al. 2022). In addition, with widespread invasions and constrained budgets, natural resource managers are frequently limited by when and where they can manage (Donlan et al. 2015; Wenger et al. 2018). Approaches for identifying effective spatiotemporal allocation of management effort remains a primary need of managers.

Predicting the effectiveness of alternative spatial allocations of management effort is challenging, yet choosing the most effective allocation is critical for successful population suppression or containment of abundant invaders (Travis and Park 2004; Eppinga et al. 2021). Various alternative spatial allocation rules of thumb have been posited for invasive species management to achieve objectives of suppressing and limiting the spread of invasive species. For example, to suppress population size, the "targeting the source" rule involves allocating management effort in locations where the invasive population is the most abundant (Baker 2017) and to limit invasive species spread, the "managing the edges" rule involves expending effort at the invasion front (Bradley 1988; Moody and Mack 1988; Bossard et al. 2000). Ultimately, however, in any given case, the optimal spatial allocation of effort will depend on management objectives, the ecology of the invader and the characteristics of the invaded system. For instance, if dispersal of the invasive species is small relative to population growth, removal at the edges of the invasion may be a more successful approach in reducing spread of an invader because efforts to remove at the core of the population may be overwhelmed by population growth (Baker 2017).

Quantitative population models are useful tools for evaluating invasive species management strategies in a virtual environment before management is implemented (García-Díaz et al. 2019; Thompson et al. 2021; Hudina et al. 2022). Simulation models can be efficiently used to compare a variety of alternatives under varying ecological and management assumptions without the substantial time and expense of on-the-ground experiments. In particular, spatially explicit population models are powerful tools for modelling population growth and spread and evaluating alternative spatial allocations of management effort (Epanchin-Niell and Hastings 2010; Bertolino et al. 2020; Goodenberger et al. 2020). Spatially explicit population models are especially useful in largescale invasion contexts to identify crucial locations wherein management resources could be directed to reduce further expansion and growth of the invasive population (Pepin et al. 2019).

A number of spatially explicit population models have been developed to evaluate spatial allocation of management effort in terrestrial invasion contexts (e.g. Epanchin-Niell et al. (2012); Baker and Bode (2016); Pepin et al. (2020)); however, such models have rarely been developed for freshwater contexts (but see Albers et al. (2018) and Kallis et al. (2023)). Spatially explicit population models in rivers are particularly difficult to parameterise due to challenges imposed by dendritic networks, including understanding individual movements (Corrales et al. 2020; Caradima et al. 2021). For instance, it is rare to observe marked individuals in aquatic systems (Ogburn et al. 2017), limiting the utility of tools such as multistate mark-recapture models (Arnason 1973) for understanding movement. In addition, software for mechanistic modelling of animal movement has primarily been developed for terrestrial systems and only recently has been advanced for modelling animal movements in rivers (Quaglietta and Porto 2019). However, as technology for studying movement continues to develop for aquatic species, spatially explicit population models may be increasingly valuable tools for informing management in river systems.

In this study, we used a spatially explicit population model to assess removal alternatives for the management of invasive rusty crayfish (*Faxonius rusticus*) in the John Day River (JDR) of Oregon, USA, a major tributary of the Columbia River. The JDR is one of the largest free-flowing rivers in the United States and holds high conservation importance as it supports a variety of salmon species of significant cultural and economic value, such as endangered spring Chinook salmon (*Oncorhynchus tshawytscha*) and the threatened steelhead (*Oncorhynchus mykiss*). The presence of rusty crayfish in the JDR remains a significant concern because they are spreading rapidly (18 km year⁻¹), reaching high local abundances (up to 50 m⁻²) and have the potential to inflict severe ecological impacts due to polytrophic and generalist feeding habits (Olden et al. 2011; Twardochleb et al. 2013).

Using a spatially explicit population model for rusty crayfish, we assessed alternative management strategies involving different spatial allocations of removal effort over a multi-year management time horizon. We evaluated the alternatives, based on performance of three management objectives that capture commonly held values of natural resource managers concerned with invasive species: suppression (minimise the overall population abundance), containment (minimise the spatial extent of invasion) and prevention (minimise spread into a particular area). The results from this study broadly seek to provide a template for the evaluation of invasive species management strategies in dendritic riverine systems.

Methods

Study system and management context

Rusty crayfish are regarded as a highly-invasive species, particularly in the JDR, due to high population growth and generalist feeding habits. Rusty crayfish have been implicated in the decline of macrophytes, aquatic insects, snails and fishes across the introduced range (Twardochleb et al. 2013). We simulated alternative strategies for managing rusty crayfish in a portion of the JDR where the species is anticipated to negatively impact the ecosystem (Falke et al. 2013; McHugh et al. 2017; Fig. 1). We divided this portion of the JDR into 35 segments of 20-km length, the maximum possible size of a 'management unit' wherein removal could be conducted, henceforth termed segments. The locations where crayfish management occurred corresponded to specific segments, selected annually (Fig. 1, Suppl. material 1: fig. S5).



Figure 1. Map of the John Day River (JDR) Basin and tributaries (Mainstem, North Fork, Middle Fork, South Fork and Murderers Creek). The dark blue region of the JDR represents the spatial extent of this study (35 segments). The light blue regions of the JDR Basin are not included in our simulations. The JDR flows into the Columbia River.

A range of management objectives are of interest to invasive species managers, including – broadly – eradication, suppression (i.e. minimising total abundance), containment (i.e. minimising range size or total spatial extent) and prevention (i.e. minimising spread into a particular geographic location, for example, the Columbia River for this study) (Gherardi et al. 2011; Rytwinski et al. 2019). Here, we focused on the latter three – suppression, containment and prevention – as eradication does not appear to be achievable, based on our results and other studies (Messager and Olden 2018).

We developed management strategies with suppression, containment, and prevention in mind (Table 1). We developed a strategy called Target Abundance primarily to address the suppression objective, with removal effort in segments with the highest total crayfish abundance. We also developed the Target Growth strategy primarily to address the suppression objective, with removal effort in segments with the highest population growth. We created the Target Edges strategy to address the containment objective, with removal effort at the invasion edges with the highest abundance and at segments adjacent to invasion edges depending on the number of segments receiving removal effort, again prioritised by abundance. An invasion edge refers to the most upstream invaded segment on the Mainstem, North Fork, Middle Fork, South Fork and Murderers Creek and the most downstream invaded segment on the Mainstem (Fig. 1). We developed the strategy Target Downstream to address both the prevention and containment objectives, with removal effort at the most downstream segments on the Mainstem with crayfish present. We also evaluated a Target Random strategy, with removal at randomly selected segments, **Table 1.** Twenty-one management alternatives that were simulated for removal of rusty crayfish in the John Day River System, including the broad management strategy, the number of segments (and percentage of the modelled system) receiving removal effort and the specific objectives targeted by the management strategy: suppression (i.e. minimise total abundance), containment (i.e. minimise total spatial extent) and prevention (i.e. minimise spread into the Columbia River). Removals were simulated to occur June through September for ten trap-nights per month. Segments receiving removal effort were selected annually given the alternative and the simulated system state.

Management Strategy	No. Segments Receiving Removal Effort (% of the JDR managed)	Objective(s) Targeted
No removals	0 (0%)	None
Target Abundance: remove at segments with highest total crayfish abundance	1 (3%), 4 (11%), 8 (23%), 16 (46%)	Suppression
Target Growth: remove at segments with highest crayfish population growth	1 (3%), 4 (11%), 8 (23%), 16 (46%)	Suppression
Target Edges: remove at edges of invasion with the highest abundance (i.e. invaded segments most downstream on the Mainstem and most upstream on the Mainstem, North Fork, Middle Fork, South Fork or Murderers Creek)	1 (3%), 4 (11%), 8 (23%), 16 (46%)	Containment
Target Downstream: remove at the most downstream segments on the Mainstem with crayfish abundance	1 (3%), 4 (11%), 8 (23%), 16 (46%)	Containment/ Prevention
Target Random: remove at randomly selected segments	1 (3%), 4 (11%), 8 (23%), 16 (46%)	Suppression/ Containment/ Prevention

with a new random selection each year. Given random selection under this strategy, segments with no crayfish could be selected for management. Finally, we evaluated a No Removals strategy to represent the status quo in the JDR.

For each of the broad management strategies (except No Removals), we simulated various levels of removal effort, which corresponded to the number of segments where removal was simulated (Table 1). We assumed removal of crayfish via trapping and physical removal, which is the most common method for crayfish (Freeman et al. 2010; Gherardi et al. 2011; Manfrin et al. 2019). We tested four levels of removal effort, such that removal was simulated at 1, 4, 8 or 16 segments out of the total 35 segments, representing approximately 3%, 11%, 23% or 45% of the modelled system (capped at the maximum feasible spatial coverage for management). Thus, with five broad removal strategies, each with four levels of effort, plus the No Removals strategy, we evaluated 21 total management alternatives. The segments selected for removal remained fixed during a year and were identified annually according to the management strategy. In addition, given high-flow conditions during the autumn and winter months that limit accessibility, we simulated removals only during June through September, with removals occurring over ten trap-nights in each of those months. We evaluated management strategies over a seven-year management time horizon.

Model structure

We developed a spatially explicit population model to simulate rusty crayfish removal, growth and movement. Our simulation model largely follows the ecological process described by Link et al. (2018) in their model for estimating abundance, growth, movement and detection efficiency using spatially referenced counts of removals from an invasive population; the primary difference is our addition of age structure. In our model, we track abundance of the population by spatial unit (i.e. a river segment) and allow the population to grow and move and to be removed as a function of removal effort. We assumed that removals occurred on ten consecutive trap-nights each month, during the months of June through September each year and that all population growth and movement occurred between each monthly removal period. The time step in the simulation model is primarily a monthly time step, but switches to a daily time step during periods of removal, when the population is otherwise closed.

Messager and Olden (2018) previously simulated the spread and removal of rusty crayfish in the JDR with a spatially explicit individual-based model. We drew many parameter values from that study, as well as the literature (see Appendix 1 for detailed parameter value descriptions). In our model, segments of 20-km length were indexed by i = 1, 2, ..., I (I = 35 segments), months were indexed by j = 1, 2, ..., I (I = 35 segments), months were indexed by k = 1, ..., K (K = 10 trap-nights). Only females were modelled and age class was indexed by a = 0, 1, 2 and 3 (where 0 = 0-1 year olds, 1 = 1-2 year olds, 2 = 2-3 year olds and 3 = older than 3). In the following sections, we describe the specific modelling structures for the removal, population growth and movement sub-models.

Removal sub-model

The removal sub-model allowed for simulation of trapping and removal of crayfish. We defined $N_{i,j,k,a}$ as the abundance at segment *i* before the k^{th} trap night during month *j*, for age *a* and $Y_{i,j,k,a}$ as the number of crayfish removed. We assumed age-0 individuals were too small to be removed by typical trapping methods (Ogle and Kret 2008). Hence, crayfish abundance and removals for age classes *a* = 1, 2, 3 and for trap nights *k* = 2,..., *K* were:

$$N_{i,j,k,a} = N_{i,j,(k-1),a} - Y_{i,j,(k-1),a}$$
(1)

$$Y_{i,j,k,a} \sim \text{Binomial}(N_{i,j,k,a},p) \tag{2}$$

with effective removal probability, *p*, modelled as:

$$p = 0.25\theta \tag{3}$$

where 0.25 indicates that a fixed 25% of the segment was covered with traps, which represents a reasonable maximum spatial coverage. We expressed θ as the probability of capture for a crayfish within the trappable area around a single trap. No information was available with which to estimate θ , so we defined a Uniform (0.1, 0.5) distribution to represent our uncertain judgment about this parameter.

The calculation of $N_{i,j,k,a}$, for j > 1 and k = 1, i.e. abundance on the first trapnight in all removal months after the first month, is described further in the movement sub-model section and initial population $N_{i,j,k,a}$ for j = 1 and k = 1 is described in the simulation study implementation section.

Population growth sub-model

After K = 10 trap nights, we calculated $R_{i,j,a}$, the population remaining after removal as:

$$R_{i,j,a} = N_{i,j,K,a} - Y_{i,j,K,a}$$
(4)

We then initiated the population growth sub-model based on $R_{i,j,a}$. Since the model was age-structured, we calculated $D_{i,j,a}$, defined as the number in the population after population growth. $D_{i,j,a}$ was based on a time-varying Leslie matrix, L_j , containing survival probabilities and fecundity rates for each age class. Since survival was applied monthly, while age transitions and reproduction occurred yearly, we created two Leslie matrices, one for all months excluding June and one for June, when age transitions and reproduction occurred. For months excluding June, L_j was:

$$\begin{pmatrix} \varphi_0 & 0 & 0 & 0 \\ 0 & \varphi_1 & 0 & 0 \\ 0 & 0 & \varphi_2 & 0 \\ 0 & 0 & 0 & \varphi_3 \end{pmatrix}$$
(5)

where φ_a were monthly survival rates for each age class (i.e. in months excluding June, population "growth" was strictly negative). In June, the population underwent age transition and reproduction and the post-breeding census matrix was:

$$\begin{pmatrix} \varphi_0 f_1 m_1 \ \varphi_1 f_2 m_2 \ \varphi_2 f_3 m_3 \ \varphi_3 f_3 m_3 \\ \varphi_0 & 0 & 0 \\ 0 & \varphi_1 & 0 & 0 \\ 0 & 0 & \varphi_2 & \varphi_3 \end{pmatrix}$$
(6)

where f_a represented age class-specific fecundity rates and m_a represented the fraction of mature females out of total females in each age class, for a = 1, 2 and 3 (Messager and Olden 2018). The first row provides each age class' contribution to the age-0 crayfish entering the population on 1 June.

We sampled rates φ_a , f_a and m_a from normal distributions. Survival rate φ_a had mean values of 0.81, 0.97, 0.94 and 0.72 with a standard deviation of 0.1 for a = 0, 1, 2 and 3, respectively and bounded between 0 and 1 (Suppl. material 1: table S3; Messager and Olden 2018). The fecundity rates, f_a , had mean values of 80, 120 and 150 with standard deviations of 10, 20 and 40 for a = 1, 2 and 3, respectively and bounded between 0 and ∞ (Suppl. material 1: fig S3; Messager and Olden 2018). The fraction of mature females, m_a , had mean values of 0.1, 0.8 and 0.9 for a = 1, 2 and 3, respectively, with a standard deviation of 0.1 and bounded between 0 and 1 (Suppl. material 1: table S3; Messager and Olden 2018). $D_{i,i,a}$ was calculated as:

$$D_{i,j,a} = L_j \times R_{i,j,a} \tag{7}$$

and rounded upwards.

We assumed that the number of crayfish in each segment could at most be 12,166,668, which was calculated as twice the maximum density (30.4 cray-fish/m²) observed in a 2016 field study for a population assumed to be at the stable age structure (Messager and Olden 2018). If the number of crayfish in a segment was greater than the carrying capacity, the excess number of cray-fish was first subtracted from age class 0 individuals, $D_{i,j,0}$, since that age class would likely be the most negatively impacted by density-dependent processes. Any remaining crayfish were subtracted from the other age classes, in order of increasing age.

Movement sub-model

After population growth, we modelled the monthly movement of crayfish between adjacent segments. We first calculated the number of crayfish that remained in each segment. The probability of staying in each segment was:

$$m_{\rm stav} = 1 - 0.5\pi (1 - u_{ii}) \tag{8}$$

In this expression, 0.5 indicates that only one half of the crayfish population in any segment was available to move because the size of a single segment was 20 km and crayfish do not disperse more than 5 km in a single month (Messager and Olden 2018). The parameter π is the probability of moving, which ranged between 0.05 and 0.25 by increments of 0.05 (Messager and Olden 2018, Suppl. material 1: table S3). Next, $u_{i,j}$ represents the proportion of crayfish that stayed in a current segment due to temperature constraints. To calculate this proportion, we obtained segment-level temperature data and calculated the fraction of days each month in which temperature was less than 6 °C, such that crayfish movement is physiologically unfeasible (Hamr 1997; Messager and Olden 2018). Hence, the term 1- $u_{i,j}$ represented the probability that crayfish were not restricted in their movement by temperature constraints. Then, for a = 1, 2 and 3, the number of crayfish that stayed in a segment was calculated as:

$$D_{i,j,a}^{\text{stay}} \sim \text{Binomial}(D_{i,j,a}, m_{\text{stay}})$$
(9)

Next, we calculated crayfish that moved downstream:

$$D_{i,j,a}^{\text{down}} \sim \text{Binomial}\left(D_{i,j,a} - D_{i,j,a}^{\text{stay}}, m_{\text{down}}\right)$$
(10)

where $D_{i,j,a} - D_{i,j,a}^{\text{stay}}$ was the number of crayfish that did not stay in segment *j* and the probability of moving downstream conditional on moving was m_{down} , which was drawn from Uniform(0.5, 1) (Messager and Olden 2018). Then, for segments not adjacent to a river fork, we calculated the number of individuals moving upstream as:

$$D_{i,j,a}^{up} = D_{i,j,a} - D_{i,j,a}^{stay} - D_{i,j,a}^{down}$$

$$\tag{11}$$

Crayfish in some segments could move upstream within the same tributary and move upstream to a new fork (i.e. segments 6, 8, 25 and 31, Suppl. material 1: fig. S5) and we needed to implement a bifurcation movement process. Due to the hydrology of the JDR, we only needed to incorporate the bifurcation process in upstream movement. In these few segments, we assumed upstream movement within the same fork and to a different fork had equal probability. Therefore, in those segments, we calculated the number of individuals that moved upstream as:

$$D_{i,j,a}^{up} = \frac{D_{i,j,a} - D_{i,j,a}^{stay} - D_{i,j,a}^{down}}{2}$$
(12)

rounded downwards and the number of crayfish that moved to a new fork as:

$$D_{i,j,a}^{\text{fork}} = D_{i,j,a} - D_{i,j,a}^{\text{stay}} - D_{i,j,a}^{\text{down}} - D_{i,j,a}^{\text{up}}$$
(13)

Finally, we redistributed crayfish in the river according to their recent movement. However, for a = 0, $D_{i,j,a} = N_{i,j,a}^{\text{redistribute}}$, since we assumed that age-0 individuals do not move (Messager and Olden 2018). Hence, we calculated the redistributed population as:

$$N_{i,j,a}^{\text{redistribute}} = D_{i,j,a}^{\text{stay}} + \sum_{h \in \text{down}_{i}} D_{h,j,a}^{\text{down}} + D_{up_{i}j,a}^{\text{up}} + D_{fork_{i}j,a}^{\text{fork}}$$
(14)

where the first term represented the population that stayed in segment *i*. The second term is the population that moved downstream into *i* from segments $h \in \text{down}_i$, where down_i was the set of segments from which crayfish could move downstream to *i*. The third term represents the number of crayfish that moved into *i* from upstream segment up_i. Finally, the fourth term is the number of crayfish that moved upstream into *i* from a segment in a different fork, fork_i (see Suppl. material 1: fig. S5 for a graphical representation of each river segment in the JDR and downstream movement directions). We assumed that, in the most upstream segments in all forks, no crayfish could move upstream out of that fork. We also assumed that crayfish in the most downstream segment in the Mainstem could move out of the JDR, which allowed us to calculate the number of crayfish that entered the Columbia River.

Once we completed the movement process, we calculated abundance at the beginning of the next month j + 1 as $N_{i,(j+1),1,a} = N_{i,j,a}^{\text{redistribute}}$. For the months June, July, August and September, removal, population growth and movement occurred and, for all other months, only population growth and movement occurred. At the end of May, before June crayfish removal, the abundance of total crayfish in a = 1, 2 and 3 (i.e. excluding a = 0) at each segment was assessed and the locations where removal would occur that June through September were informed by the simulated management strategy.

Simulation study implementation

Population simulations were coded in R (R version 4.3.1, R Core Team 2023). We simulated each of the 21 management alternatives under the same 20 parameter sets to account for parametric uncertainty for each parameter (e.g. survival, fecundity and movement rates) and ran 50 simulations for each parameter set to account for stochasticity. To create the parameter sets, we performed 20 independent draws from the parametric distributions provided in the model descriptions (Suppl. material 1: table S3).

Each simulation under each alternative was initialised with the same segment-level population, which was informed by an intensive crayfish survey in 2016 (Suppl. material 1: fig. S1, Messager and Olden 2018). Since rusty crayfish were likely introduced to the JDR in 1999, we assumed that, by 2016, the population had reached a stable age distribution and for each parameter set we calculated an annual Leslie matrix and then calculated the stable age distribution as the eigenvector associated with the largest eigenvalue of the annual Leslie matrix:

$$\begin{pmatrix} \varphi_0^{12} f_1 m_1 \ \varphi_1^{12} f_2 m_2 \ \varphi_2^{12} f_3 m_3 \ \varphi_3^{12} f_3 m_3 \\ \varphi_0^{12} & 0 & 0 \\ 0 & \varphi_1^{12} & 0 & 0 \\ 0 & 0 & \varphi_2^{12} & \varphi_3^{12} \end{pmatrix}$$
(15)

Therefore, although the segment-level population was the same across parameter sets, the distribution of each age class at each segment differed between parameter sets.

Evaluation of alternatives

We evaluated the performance on each objective – suppression, containment and prevention – under each alternative. We only considered adults (a = 1, 2and 3) in our calculation of management outcomes because of the demonstrated ecological effects of adult crayfish. We expressed management outcomes for the suppression objective for each simulation as the total crayfish population size after 7 years of management (month J = 84), $\sum_{i=1}^{N} \sum_{j=1}^{N_{i,j,a}^{\text{redistribute}}} \sum_{j=$ of minimising this quantity); the number of years since the last extensive survey of rusty crayfish in the JDR. We expressed management outcomes under the containment objective for each simulation as the proportion of segments in which rusty crayfish abundance exceeded a threshold after 7 years of management (with the objective of minimising this quantity). We defined this threshold as 10% of the average abundance for a = 1, 2 and 3 under the No Removals strategy and assumed that a segment-specific abundance below this threshold would represent functional eradication (sensu Green and Grosholz (2021)) in this system and values above this threshold would indicate the segment had an ecologically impactful crayfish population. We expressed management outcomes under the prevention objective for each simulation as the cumulative number of crayfish that moved out of the JDR and entered the Columbia River (with the objective of minimising this quantity). This was calculated as $\sum_{i,j,a} D_{i,j,a}^{\text{down}}$ for the most downstream segment i in the Mainstem of the JDR (i = 22, Suppl. material 1: fig. S5).

We considered two decision criteria: expected value and mini-max. The expected value criterion, used for risk-neutral decision-makers, selects for the management alternative with the best expected performance (i.e. average simulated value) over simulations. The mini-max criterion is a risk-averse decision criterion that selects for the alternative that minimises the maximum possible loss given uncertainty (i.e. the worst outcome over all simulations) (Savage 1951).

Multiple objective assessment

Multiple objective decisions are common in natural resources management (Converse 2020) and we were interested in evaluating strategies across our three objectives simultaneously. Pareto-efficient alternatives, or non-dominated alternatives, are the alternatives within a set under which the outcome on one objective cannot be improved without a reduction in another objective (Cohon 1978; Kennedy et al. 2008). Non-optimal alternatives, or dominated alternatives, are clearly inferior because some other alternative in the set performs at least as well as the dominated alternative on all objectives and performs strictly better on at least one. We found the Pareto front, the set of Pareto-efficient alternatives, using both the expected value and mini-max criteria, across our three objectives: suppression, containment and prevention. This is the set of alternatives that strikes some efficient trade-off between suppressing the population, containing it and preventing it from entering the Columbia River.

Results

The Target Abundance strategy performed best on the suppression objective with respect to expected value, regardless of the number of segments receiving removal effort (Fig. 2A, Table 2). The final population distribution further revealed that this strategy led to overall population suppression in the JDR (Fig. 3; with 16 segments of removal effort; see Suppl. material 1: fig. S2 for other segments receiving removal effort). However, this strategy was not optimal under the risk-averse mini-max criterion for the suppression objective (Table 3). In addition, the Target Abundance performed poorly on the containment and prevention objectives across all levels of removal effort (Tables 2, 3).

The Target Growth strategy, across all levels of removal effort, performed best on the mini-max criterion for the suppression objective (Table 3), yet was the worst in terms of expected value (Table 2, Fig. 2A). Target Growth had the best expected value performance for the containment objective given one segment of removal effort and was the second-best strategy for this objective given 4, 8 or 16 segments of effort (Table 2). This strategy was never optimal with respect to the prevention objective given either the expected value or the mini-max criteria and any level of removal effort (Tables 2, 3).

The Target Edges strategy performed poorly on the suppression objective across all levels of removal effort (Tables 2, 3). However, with 16 segments of effort, this strategy did suppress crayfish population at the "edges" of invasion, as there were low final populations in all major tributaries of the JDR and in the most upstream and downstream segments of the Mainstem of the JDR (Fig. 3). Therefore, this strategy had the best expected value for the containment objective for 16 segments of effort (Table 2, Fig. 2B).

Target Downstream was the best strategy on the prevention objective regardless of the decision criterion and regardless of the number of segments of removal effort (Tables 2, 3, Figs 2C, 3). In addition, this strategy performed the best in terms of expected value for the containment objective for 4 or 8 segments of effort (Table 2). Overall, the Target Downstream strategy did not perform well on the suppression objective (Tables 2, 3).

The Target Random strategy, in terms of the mini-max criterion, was the worst strategy for both suppression and prevention objectives and performed equally as bad as all other strategies on the containment objective, across all numbers of segments receiving removal effort (Table 3). Compared to other strategies, Target Random was neither the optimal nor the worst strategy in terms of the expected value criterion across all objectives. Overall, the Target Random strategy had variable outcomes on all objectives, as shown by the various outlier values (Fig. 2). However, Target Random did perform better than No removals. Overall, the No removals strategy performed the worst across every objective and criterion (Tables 2, 3).

The Pareto efficient strategies, in terms of expected value, included Target Abundance, Target Downstream and Target Random across all levels of removal effort (Table 2). For 4 and 8 segments of effort, the dominated strategies (Target Growth and Target Edges) were dominated by the Target Downstream strategy and, for 16 segments of effort, the dominated strategy (Target Growth) was dominated by Target Edges (Table 2). In terms of the mini-max criterion, Target Growth and Target Downstream were Pareto efficient regardless of the level of removal effort (Table 3). For 4, 8 and 16 segments of effort, the Target Random strategy was dominated by every other strategy (Table 3).



Figure 2. Boxplots displaying the performance of each crayfish removal alternative, except No Removals, across all parameter sets and simulations for each objective **A** suppression: final total crayfish abundance (millions) **B** containment: percent invaded and **C** prevention: total crayfish in the Columbia River (millions). The horizontal black dotted line represents the expected value outcome under No Removals. In each boxplot, the red line is the mean, the black line is the median and the red point is the maximum value. In subfigures **A**–**C**, the facet plots represent 1, 4, 8 and 16 segments receiving removal effort. We express strategies Target Abundance as Abundance, Target Growth as Growth, Target Edges as Edges, Target Downstream as Downstream and Target Random as Random.

Since all the objectives were based on either final population abundance (suppression), final distribution (containment) or cumulative abundance (prevention), we did not focus on changes in the population over time. However, under all alternatives, the abundance of crayfish slightly increased over time (Suppl. material 1: figs S3, S4).



= 1 to 100,000

- 100,000 to 200,000
- 200,000 to 400,000
- 400,000 to 600,000
- 600,000 to 800,000
- 800,000 to 1,000,000

Figure 3. Segment-level total crayfish abundance after 7 years of management, averaged across simulations and parameter sets for each strategy (No removal, Target Abundance, Target Growth, Target Edges, Target Downstream and Target Random) with 16 segments of removal effort. The colours show segment level abundance.

Table 2. Consequence table of simulation results for rusty crayfish (Faxonius rusticus) removal in the John Day River, Oregon, USA, based on the expected value decision criterion. The first column indicates the alternative and the second to fourth columns represent the expected value for that alternative under each of three objectives, with M representing millions of crayfish. The bold text within a cell represent the minimum (i.e. preferred) expected value for each objective, for a given number of segments receiving removal effort. The fifth column indicates the alternative, if any, that dominated the alternative in the row, again for a given number of segments receiving removal effort. An alternative is Pareto efficient if no alternative dominates that alternative, indicated with "None". We express strategies Target Abundance as Abundance, Target Growth as Growth, Target Edges as Edges, Target Downstream as Downstream and Target Random as Random.

Alternative management strategy,	0	Dominated by X Alternative			
no. segments of removal effort	Suppression (in millions)	Containment (%)	Prevention (in millions)		
No removals, 0	21.13 M	90.3%	1.15 M	None	
Abundance, 1	20.52 M	90.2%	1.15 M	None	
Growth, 1	20.83 M	89.7%	1.15 M	None	
Edges, 1	20.68 M	90.0%	0.83 M	None	
Downstream, 1	20.81 M	90.1%	0.48 M	None	
Random, 1	20.61 M	90.0%	1.10 M	None	
Abundance, 4	18.82 M	89.6%	1.14 M	None	
Growth, 4	20.05 M	87.2%	1.01 M	Downstream, 4	
Edges, 4	19.24 M	88.1%	0.48 M	None	
Downstream, 4	19.37 M	86.2%	0.18 M	None	
Random, 4	19.00 M	88.6%	0.96 M	None	
Abundance, 8	16.67 M	85.7%	1.02 M	None	
Growth, 8	18.34 M	83.1%	0.58 M	Downstream, 8	
Edges, 8	17.92 M	85.1%	0.31 M	Downstream, 8	
Downstream, 8	17.32 M	81.4%	0.15 M	None	
Random, 8	16.93 M	85.7%	0.83 M	None	
Abundance, 16	11.81 M	74.1%	0.67 M	None	
Growth, 16	14.25 M	72.9%	0.22 M	Edges, 16	
Edges, 16	14.24 M	71.4%	0.22 M	None	
Downstream, 16	13.17 M	73.7%	0.15 M	None	
Random, 16	12.78 M	78.3%	0.56 M	None	

Table 3. Consequence table of simulation results for rusty crayfish (*Faxonius rusticus*) removal in the John Day River, Oregon, USA, based on the mini-max decision criterion. The first column indicates the alternative and the second to fourth columns represent the maximum predicted value for that alternative under each of three objectives, with M representing millions of crayfish. The bold and underlined text within a cell represent the minimum (i.e. preferred) of the maximum values for each objective, for a given number of segments receiving removal effort. The fifth column indicates the alternative, if any, that dominated the alternative in the row, again for a given number of segments receiving removal effort. An alternative is Pareto efficient if no alternative dominates that alternative, indicated with "None". We express strategies Target Abundance as Abundance, Target Growth as Growth, Target Edges as Edges, Target Downstream as Downstream and Target Random as Random.

A14	Objective (expected value)				
no. segments of removal effort	Suppression (in millions)	Containment (%)	Prevention (in millions)	Dominated by X Alternative(s)	
No removals, 0	80.30 M	100%	5.73 M	N/A	
Abundance, 1	79.10 M	100%	5.72 M	None	
Growth, 1	78.72 M	100%	5.73 M	None	
Edges, 1	79.80 M	100%	3.78 M	Downstream, 1	
Downstream, 1	79.58 M	100%	2.45 M	None	
Random, 1	79.91 M	100%	5.72 M	Abundance, 1 & Downstream, 1	
Abundance, 4	75.77 M	100%	5.72 M	Growth, 4 & Edges, 4	
Growth, 4	74.68 M	100%	5.31 M	None	
Edges, 4	75.52 M	100%	2.45 M	None	
Downstream, 4	76.11 M	100%	1.76 M	None	
Random, 4	76.84 M	100%	5.72 M	All	
Abundance, 8	72.32 M	100%	4.53 M	Edges, 8 & Downstream, 8	
Growth, 8	69.91 M	100%	5.00 M	None	
Edges, 8	70.08 M	100%	2.10 M	None	
Downstream, 8	71.49 M	100%	1.70 M	None	
Random, 8	73.30 M	100%	5.72 M	All	
Abundance, 16	63.04 M	100%	3.54 M	Growth, 16 & Edges, 16 & Downstream, 16	
Growth, 16	59.64 M	100%	1.75 M	None	
Edges, 16	59.80 M	100%	1.85 M	Growth, 16	
Downstream, 16	62.13 M	100%	1.70 M	None	
Random, 16	63.61 M	100%	4.61 M	All	

Discussion

We used a spatially explicit population model to simulate rusty crayfish population growth, movement and removal in the JDR and evaluated different management strategies across various effort levels (i.e. number of locations receiving management). We evaluated the performance of all alternatives in meeting objectives of suppression (i.e. minimise the overall population size or total abundance of rusty crayfish), containment (i.e. minimise the range size or spatial extent of rusty crayfish in the JDR) and prevention (i.e. minimise the number of crayfish entering the Columbia River). Our results point to three major outcomes with respect to comparing spatially explicit management alternatives for an invasive species.

First, all strategies involving removal of invasive crayfish performed better than No Removals on every objective in terms of both decision criteria, yet the optimal strategy often varied by objective, decision criteria or the level of removal effort. For example, if the prevention objective was the priority, the Target Abundance strategy would be preferred for a risk-neutral decision-maker (expected value decision criterion), but for a risk-averse decision-maker (mini-max criterion), the Target Growth strategy would be preferred (Tables 2, 3, Fig. 2). The optimal strategy for the containment objective in terms of the expected value criterion varied across the number of segments that received management (Table 2). However, Target Downstream was consistently the best strategy for the prevention objective across all numbers of segments receiving management and across the two decision criteria, yet this strategy performed poorly on the suppression objective (Tables 2, 3, Fig. 2). In addition, for this objective, removing at one segment under the Target Downstream strategy performed better than any other strategy with four segments of removal effort (Table 2). The Target Downstream strategy is similar to other efforts in freshwater invasive species management to reduce spread rates into an uninvaded area (Rytwinski et al. 2019). For example, to minimise stone moroko (Pseudorasbora parva) spread in England and Wales, resource managers targeted management at lakes that were located on the floodplain (Britton et al. 2011). In addition, a study of invasive signal crayfish (Pacifastacus leniusculus) in Europe revealed that removing crayfish at the leading front of the invasion may delay colonisation to new areas (Moorhouse and Macdonald 2011).

Second, because no single management strategy performed the best across every objective and decision criterion, trade-offs amongst objectives are unavoidable. We found that the Target Downstream strategy was the only strategy that was Pareto efficient (i.e. not dominated by another strategy) regardless of the number of segments receiving removal effort and regardless of the decision criterion (Tables 2, 3, Fig. 2). This is because the Target Downstream strategy was always the best strategy for the prevention objective. This result differs from findings in terrestrial invasive species contexts that suggest targeting the core of invasion (e.g. Baker (2017); Lustig et al. (2019)), equivalent to the Target Abundance strategy here, is most effective. However, our result is similar to findings for invasive weed management, in which targeting outlier invasions can be effective (Bossard et al. 2000). In many invasive species management contexts, preventing spread into a new area is an important objective, especially when eradication is not feasible (Green and Grosholz 2021). For example, a primary objective of bigheaded carp (Hypophthalmichthys spp.) management in the mid-western United States is to minimise spread into the Great Lakes (MacNamara et al. 2016). In addition, the U.S. Forest Service developed a campaign for invasive spongy moth (Lymantria dispar) called "Slow the Spread" to minimise new invasions (Grayson and Johnson 2018). Hence, for a widespread invasion, management strategies that best prevent spread into new areas may be preferred. However, when managers make decisions regarding spatial management, it is important to acknowledge that some strategies may not be mutually exclusive and management locations may overlap (e.g. target growth and target edges strategies may involve removal at the same locations).

Third, as expected, it is better to conduct management at a higher intensity. For example, the expected value outcome of the suppression objective under the Target Abundance strategy showed a 3% improvement comparing No removals to one segment receiving management, an 8% improvement comparing 1–4 segments managed, an 11% improvement comparing 4–8 segments managed and a 29% improvement when comparing 8–16 segments receiving management. In general, the outcomes of the best alternative under all objectives and decision criteria improved with increasing management intensity (Tables 2, 3). A variety of invasive crayfish management studies have also recognised that higher intensity removals

achieve better management results (Hansen et al. 2013; Perales et al. 2021; Reisinger et al. 2024). For example, a whole lake invasive rusty crayfish removal study in Wisconsin, USA, showed that intensive trap efforts could suppress rusty crayfish populations (Hein et al. 2007). However, although intensive crayfish removal may effectively suppress populations, especially in closed systems, these intensive efforts often come at a significant economic cost and are increasingly challenging to conduct in open systems like rivers (Fausch and García-Berthou 2013).

Cost is often a major consideration in management (Epanchin-Niell 2017). To account for this, we considered alternatives with varying intensities (i.e. number of segments with removal effort) as a proxy for cost. Implementing a "proxy" for cost is a simple and effective way to help identify the degree to which management outcomes may improve if budget is increased. Here, we showed that increasing the number of segments that received management greatly improved outcomes in suppressing the population, yet there were only marginal benefits in improving outcomes for the prevention objective (Fig. 2).

We relied on simulations of a population model to conduct this study and, with all such studies, there are some limitations to acknowledge. In terms of the ecology of rusty crayfish, in our model, temperature was the only covariate included, but other environmental factors may be important. For example, river flow results in variability in dispersal rates of crayfish (Ehrlén and Morris 2015), though it would be challenging to simulate at the scale of our study. In addition, we assumed that every location was available for removal, which may not be the case in reality due to restrictions in access and this assumption may impact management outcomes. For instance, Bertolino et al. (2021) showed that restricting access of natural resource managers to private property can cause delays and lead to more costly management. In addition, our model assumed a high level of trap coverage in each segment and various levels of trap coverage could be evaluated in future studies. Finally, the only management technique we considered involved removal of adult crayfish (i.e. older than 1 year old) via physical trapping. Removal techniques involving juvenile crayfish are rare and difficult to implement. Invasive crayfish management programmes will benefit from studies that identify novel, effective techniques (e.g. biocides, Manfrin et al. (2019)).

We selected segments for management, based on perfect knowledge of the system, which will not be the case in real applications. If we do not have perfect knowledge of the state of the system, we would need to rely on monitoring data to identify removal segments, allowing for the implementation of dynamic or adaptive management (Lyons et al. 2008; Williams and Brown 2014). We attempted to create this case study in an adaptive management context, which relied on creating a Bayesian estimation model for abundance. However, we were unable to produce reasonably accurate estimates, likely because we were collecting data from only a subset of segments in only four months of the year. If management were to occur more frequently or additional data streams were integrated (e.g. detection-non/ detection or radio telemetry data), an adaptive management framework may be appropriate, as abundance and other parameters could be better estimated. However, our results provide insight into the management strategies that may be most effective, as well as their overall effectiveness in the best-case scenario, when knowledge about the system is perfect.

The JDR represents a particularly challenging system, in terms of the extent of the basin and of the crayfish invasion, making it difficult to accurately manage or monitor the entire basin. While managers are not currently removing crayfish in the JDR, we provide context on the potential effects of management which could be used by future decision-makers. Although we showed that removing crayfish resulted in better management outcomes than No Removals, on average, no strategies resulted in eradication, successful containment or prevention of crayfish moving downstream into the Columbia River. Therefore, rusty crayfish invasion in the JDR can serve as a precautionary tale, as management outcomes would have likely improved if management had begun earlier in the invasion process (Messager and Olden 2018). Therefore, we emphasise the value of early detection and rapid response for minimising the impacts of invasive species before the invasion becomes too large for management to be effective (Reaser et al. 2020).

In conclusion, we provided an approach to simulate an aquatic invasive species in a complex riverine environment. In general, there are very few applications of population models that evaluate spatially explicit management in riverine contexts (Corrales et al. 2020). We described a flexible modelling framework that integrated different spatial management strategies and is broadly applicable to different species and regions of interest. Spatially explicit population models can offer natural resource managers a cost effective tool to examine management alternatives.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: BKT, JDO, SJC. Data curation: JDO. Formal analysis: BKT. Funding acquisition: JDO, SJC. Investigation: SJC, JDO, BKT. Methodology: SJC, BKT, JDO. Supervision: SJC, JDO. Validation: JDO, SJC. Visualization: JDO, SJC, BKT. Writing - original draft: BKT. Writing - review and editing: JDO, SJC, BKT.

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Data availability

R scripts and data sources underpinning the analysis of this paper are deposited on GitHub at: https://github.com/Quantitative-Conservation-Lab/Thompson_etal_2024_NeoBiota.

All result files can be found at: https://doi.org/10.5281/zenodo.12761044. References to parameter values used in the model and supplemental figures can be found in Suppl. material 1.

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Supplementary material 1

Additional details of the crayfish model and supplemental figures

Authors: Brielle K. Thompson, Julian D. Olden, Sarah J. Converse Data type: docx

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Research Article

Invasion and spread of the neotropical leafhopper *Curtara insularis* (Hemiptera, Cicadellidae) in Africa and North America and the role of high-altitude windborne migration in invasive insects

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Abstract

Invasive insects threaten ecosystem stability, public health, and food security. Documenting newly invasive species and understanding how they reach into new territories, establish populations, and interact with other species remain vitally important. Here, we report on the invasion of the South American leafhopper, Curtara insularis into Africa, where it has established populations in Ghana, encroaching inland at least 350 km off the coast. Importantly, 80% of the specimens collected were intercepted between 160 and 190 m above ground. Further, the fraction of this species among all insects collected was also higher at altitude, demonstrating its propensity to engage in high-altitude windborne dispersal. Its aerial densities at altitude translate into millions of migrants/km over a year, representing massive propagule pressure. Given the predominant southwesterly winds, these sightings suggest an introduction of C. insularis into at least one of the Gulf of Guinea ports. To assess the contribution of windborne dispersal to its spread in a new territory, we examine records of C. insularis range-expansion in the USA. Reported first in 2004 from central Florida, it reached north Florida (Panhandle) by 2008-2011 and subsequently spread across the southeastern and south-central US. Its expansion fits a "diffusion-like" process with 200-300 km long "annual displacement steps"—a pattern consistent with autonomous dispersal rather than vehicular transport. Most "steps" are consistent with common wind trajectories from the nearest documented population, assuming 2-8 hours of wind-assisted flight at altitude. Curtara insularis Citation: Nartey R, Chamorro L, Buffington M, Afrane YA, Mohammed AR, Owusu-Asenso CM, Akosah-Brempong G, Pambit-Zong CM, Hendrix SV, Dao A, Yaro AS, Diallo M, Sanogo ZL, Djibril S, Halbert SE, Bamou R, Nance CE, Bartlett CR, Reynolds DR, Chapman JW, Obiri-Danso K, Lehmann T (2024) Invasion and spread of the neotropical leafhopper Curtara insularis (Hemiptera, Cicadellidae) in Africa and North America and the role of highaltitude windborne migration in invasive insects. NeoBiota 96: 173-189. https://doi. org/10.3897/neobiota.96.130615 has been intercepted at US ports and on trucks. Thus, it uses multiple dispersal modalities, yet its rapid overland spread is better explained by its massive propagule pressure linked with its highaltitude windborne dispersal. We propose that high-altitude windborne dispersal is common yet under-appreciated in invasive insect species.

Key words: Africa, high-altitude windborne dispersal, invasive species, leafhopper, range-expansion, vehicular transport

Introduction

Invasive insect species pose extreme threat to biodiversity, ecosystem stability, and human welfare, as many invasives impact public health (e.g., Aedes aegypti, Yellow Fever virus, Anopheles stephensi), and food security (e.g., Ceratitis capitata, Helicoverpa armigera; (Elton 1958; Mack et al. 2000; Lounibos 2002; Hulme et al. 2008; Jones et al. 2019; Takken and Lindsay 2019; Li et al. 2020; Pysek et al. 2020; Sinka et al. 2020; Turner et al. 2021; WHO 2023). Documenting invasive species and understanding how they reach new territories, establish populations, and interact with other species remain vitally important despite limited success in reversing invasions after establishment of populations in new regions (Elton 1958; Mack et al. 2000; Lounibos 2002; Hulme et al. 2008; Anderson et al. 2010; Lehmann et al. 2023). Typically, dispersal of invasive species is divided into (i) long-range movements, e.g., between continents (ii) long-range spread post-arrival in a new territory, and iii) local short-range spread within and between adjacent habitats. Most attention is focused on cross-continent movements (i) because prevention at this stage would be most effective, yet the 'secondary spread' (ii) determines whether the species remains stable near its introduction site ("alien" or "naturalized") vs. "invasive." High dispersal capacity is a key trait for invasive species (Mack and Occhipinti 1999; Hulme et al. 2008; Tsoar et al. 2011; Jones et al. 2019). In most insects, these dispersal modalities are believed to be mediated by vehicular transport, especially via the maritime trade (Elton 1958; Lounibos 2002; Hulme et al. 2008; Renault et al. 2018; Turner et al. 2021). However, apart from a few major pests, the dearth of information concerning the majority of introduced insects and inherent bias in our methodologies and expectations may limit understanding of dispersal modalities in invasive species.

Sampling of insects at altitude has often been focused on particular pests such as the desert locusts, the brown planthopper, armyworm moths, and malaria mosquitoes, yet these studies revealed surprising diversity and abundance of insects (Glick 1939; Riley et al. 1995; Reynolds et al. 1996; Riley and Reynolds 1996; Chapman et al. 2004; Anderson et al. 2010; Drake and Reynolds 2012; Hu et al. 2016; Florio et al. 2020; Wu et al. 2021; Yaro et al. 2022 ; Huang et al. 2024). Many of these windborne-migrant insects have been implicated to cover tens, hundreds and thousands of kilometers in their journey (Ghauri 1983; Drake and Reynolds 2012; Fu et al. 2014; Chapman et al. 2015; Hu et al. 2016; Wu et al. 2021).

The interception of the leafhopper *Curtara insularis* (Caldwell, 1952; Suppl. material 2: fig. S1) flying at altitude, representing a new continental record for Africa, suggests that high-altitude windborne dispersal plays a key role in the species' rapid spread post-arrival into the continent. A member of a genus that is endemic to the Western Hemisphere, *C. insularis* was originally known from Argentina, Paraguay, and Brazil (Zahniser and Nartey 2024). It has expanded across the Americas since the early 2000s (Kittelberger et al. 2021; Zahniser and Nartey 2024). Unlike certain members of its family, *C. insularis* is not known to vector plant pathogens or impact any crop. Nonetheless, as it feeds on new host plants in its new range, it might play a new role as a vector of local pathogens. An example of such a case is the glassy-winged sharpshooter (*Homalodisca vitripennis* (Germar)) that has changed transmission patterns of local strains of the plant-pathogenic bacterium, *Xylella fastidiosa*, in its new range, i.e., the Western USA, resulting in epidemics of oleander leaf scorch and Pierce's disease in southern California (Almeida and Nunney 2015; Pysek et al. 2020).

Typically, the rapid spread of invasive insects is attributed to vehicular transport, which is often involved to some or great extent (below). However, here, we assess the role of high-altitude windborne dispersal in the spread of an invasive insect over a new territory. We present results from our aerial and ground-level surveillance in Africa as well as an analysis of a new compilation of distributional records of *C. insularis* in the US based on multiple data sources including citizen-science databases. We offer a descriptive, semiquantitative framework to ascertain the relative contribution of windborne spread versus vehicular spread using spatio-temporal records and data on wind patterns. Based on our results, we propose that high-altitude windborne dispersal is especially common in many invasive insect species, in the hope it would be subject to a rigorous test in the near future.

Methods

Sampling sites

Aerial collection stations were established in rural open areas in Ghana and Mali (Fig. 1A). In southern Ghana two stations were set up in the moist-semi-deciduous forest near the town of Agogo (6.961, -0.960), and in the Guinea woodland ecozone near the town of Wenchi (7.781, -2.162). In southern Mali one station was set in the Sudano-Guinean ecozone near the village Kenieroba (12.112, -8.332). Agogo, Wenchi, and Kenieroba receive 1200–1400, 1000–1200, and 900–1000 mm of rain annually, respectively. However, in Kenieroba, the rainy season is confined to May-November whereas the Ghanian sites receive rains most months of the year (Siebert 2014). These areas are interspersed with farmland along rivers and grassland. The main crops grown near Agogo are cocoa, coffee, oil palm, citrus, cashew, mango, cassava, yam, among others. The main crops in Wenchi are corn, rice, sorghum, yam, plantain, groundnuts, and cowpea, among others. In Kenieroba, farmers grow mainly rice, sorghum, millet, corn, beans, among other crops; cereals are grown during the wet season (May-October) and vegetables in smaller plots during dry season (November-April).

Aerial and ground-level insect collection and specimen processing

The aerial collection methods were described in detail previously (Huestis et al. 2019; Florio et al. 2020), Briefly, insect sampling was conducted using sticky nets (panels, each 1 m wide \times 3 m long) attached to the tethering line of 3 m diameter helium-filled balloons. Each balloon typically carried three panels, set at 120 m, 160 m, and 190 m agl (above ground level). Balloons were launched around 17:30

before sunset and retrieved around 07:00, after sunrise the following morning. To control for insects trapped near the ground as the panels were raised and lowered, comparable control panels were raised up to 100 m agl and immediately retrieved during each balloon launch and retrieval operation. Following panel retrieval, inspection for insects was conducted in a dedicated clean area. Individual insects were removed from the nets with forceps, counted, and stored in vials containing 80% ethanol. Concurrent samples were collected from a 1 m² panel mounted 1 m above ground to represent ground insects. This net was attached to a line from a frame in such a way that allowed it to orient perpendicular to the wind direction like the nets suspended from the helium balloon.

Taxon identification

Using a dissecting microscope, African specimens were identified morphologically to order and to morphospecies, counted, and recorded. Specimens of the selected morphotypes were identified by expert taxonomist who narrowed the identification down to species or genus. All African specimens of *C. insularis* were confirmed by Dr. James Zahniser (USDA-APHIS, National Museum of Natural History [USNM], Smithsonian Institution, Washington, DC, USA). Voucher specimens are deposited at USNM.

Distribution data in the USA

Publicly available records of observations of *C. insularis* from BugGuide.net (n = 22; (BugGuide 2024)), iNaturalist (n = 827; (iNaturalist 2024)) that met "Research grade" standard (verified by SVH) were downloaded and added to records available from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI) (N = 266) after the specimens were identified by FDACS-DPI hemipterists. Vouchers for FDACS-DPI specimens are deposited in the Florida State Collection of Arthropods. The data from Florida also included 3 interception records in trucks that originated from Arizona (2) and Mexico (1). Border interception records of *C. insularis* (since 2019) from ports of entry in the USA (identified by Dr. James Zahniser, USDA) were provided from USDA/APHIS (N = 9) included the port of entry and date of collection. Of 1,124 observations collected until December 31, 2023, from the USA, 11 represented insects during transit, and thus were excluded from distribution data that were used to plot *C. insularis* range in the USA. This compilation of data is included in Suppl. material 1.

Data analysis

Subsamples of the Mali collections from March to December 2019 and the Ghana collections from May to October 2021 were evaluated for the presence of *C. insularis*. Every month of collection at each study site was represented by at least 6 panels at altitude and at least 4 panels at ground level. The total number of insects per panel represents the 'panel density'. Aerial density was estimated as the panel density of the species divided by the total air volume that passed through that net that night (i.e., aerial density = panel density/volume of air sampled, and volume of air sampled = panel surface area × mean nightly wind speed × sampling duration). The panel surface area was 3 m². Wind-speed data were obtained from the atmospheric re-analyses of the global climate (ERA5). Hourly data consistent of the eastward and northward components (horizontal vectors) of the wind were available at 31-km surface resolution at 2 and 300 m agl (1000 and 975 mbar pressure levels). Overnight records (19:00 through to 06:00) were averaged to calculate the nightly mean direction and mean wind speed over each African sampling station and select locations in the USA (below) based on standard formulae using code written in SAS (SAS software 2019).

The intensity of migration was expressed as the expected number of migrants crossing a line of 1 km perpendicular to the wind direction at altitude, which reflect their direction of movement (Drake and Reynolds 2012; Hu et al. 2016; Reynolds et al. 2017; Florio et al. 2020). We used the mean wind speed at altitude during the migration season (Table 1) and assumed that the leafhoppers fly in a layer depth of 200 m above ground level (Florio et al. 2020). The nightly migration intensity was computed across the flight season (including sampling nights during which no migrants were captured). The corresponding annual index was estimated by multiplying the nightly index by the period of migration, estimated from the first and last month that the species was captured.

To assess the likelihood of windborne movement to a new locations in the USA, we identified new sites where *C. insularis* was observed for the first time outside its previous year's range, defined by connecting all the extreme points of its cumulative distribution in the previous year. For each new site, we consider its nearest known site—where *C. insularis* was previously reported—as a putative source. Underlying our approach is the assumption that the missing data due to low sampling in certain localities and/or certain years would generate noisier data-patterns rather than as systematic pattern. Therefore, finding a consistent biological trend (signal) in these data, relevant to the process in question is likely produced by a biological process rather than by variation in sampling intensity. Virtually all datasets on geographic expansion at these scales of time and space would present similar "imperfections", inviting inquiries to better assess and address their limitations. However, large Citizen-Scientist databases provide compelling advantages as pointed out by (Kittelberger et al. 2021).

The annual distributions of nightly (19:00–06:00) winds during the year of the new record were plotted as vectors pointing to the direction the insects would be carried if they flew 8 hours from that site on that night's wind at 300 m agl. The self-propelled flight speed of leafhoppers does not typically exceed 1 m s⁻¹ (Zhou et al. 2003) and therefore at altitude with winds exceeding 4 m/s, the direction of the movement of the insects will be determined primarily by the wind. Only nights with at least 8 hours when temperatures were > 16 °C at altitude and on the ground were included (assuming no flight occurred below 16°C (Shields and Testa 1999). The total flight duration of tethered leafhoppers reached over 7 h (Zhou et al. 2003) and additionally, inferences about windborne leafhoppers in the US traveling hundreds of kilometers per night (or tens of kilometers/night over successive nights) have been made based on their phenology (Carlson et al. 1992; Shields and Testa 1999; Reynolds et al. 2017). Thus, we assumed that C. insularis could fly at altitude between 2 and 8 h per night. The wind trajectories were projected on maps based on the wind direction, speed, and 8 hours of flight using SAS 9.4 (SAS software 2019). Windborne dispersal from the putative source to its destinations was assessed based on visual inspection of the trajectory distributions.

	Agogo (panels: 66/19) ª		Wenchi (panels: 57/26)		Kenieroba (panels: 47/0)	
	Altitude (26) ^b	Ground (21) ^b	Altitude (19)	Ground (12)	Altitude (35)	Kenieroba (panels: 47/0)
Panel density C. insularis ^c	0.23 (0-0.47)	0.05 (0-0.15)	0.40 (0.0-0.84)	0.17 (0-0.53)	0	0
Total C. insularis / total insects ^d	0.26 (6/2314)	0.06 (1/1662)	0.21 (8/3852)	0.17 (2/1193)	0 (0/3348)	0% (0/549)
Aerial density C. insularise	0.41 (0-0.83)	nd	1.0 (0-2.08)	nd	0	0
Dispersal mass (n/[km night] ^f	17,004 (0-34,422)	nd	35,424 (0-73,682)	nd	0	0
Wind speed (m/s) ^g	4.75 (4.6-4.9)	1.86 (1.8–1.9)	4.07 (3.96-4.18)	1.85 (1.8–1.9)	1.91 (1.8–2.0)	1.91 (1.8–2.0)
Wind direction (°) ^g	244 (243.7–244.2)	246.3 (246.1–246.6)	244.2 (244.0–244.5)	246.8 (246.5–247.2)	229.2 (229.0–229.4)	229.1 (229.0–229.4)
Temperature (°C) ^g	23.9 (23.8–24.1) [19.3]	25.3 (25.1–25.4) [20.7]	24.9 (24.7–25.1) [19.9]	26.4 (26.2–26.5) [21.3]	28.0 (27.6–28.4) [17.6]	29.5 (29.1–29.9) [19.5]]
Relative humidity (%) ^g	85.0 (84.1-85.8)	84.1 (83.2-84.9)	80.2 (79.2-81.2)	80.8 (79.8-81.8)	75.6 (73.5–77.7)	75.6 (73.5–77.7)

Table 1. Mean panel and aerial densities of *C. insularis* at altitude and ground levels across study sites with average temperature, humidity, wind speed, and direction during the migration period (May-August).

^a Total number of panels/control panels inspected.

^b Number of panels.

^c Mean (95%CI) panel density of *C. insularis* /panelr during May-September (zeros replace negative lower 95%CL).

^d Percentage of *C. insularis* from total insects (% total *C. insularis*/total insects).

^c Mean (95%CI) aerial density of *C. insularis* /10⁶ m³ of air during May-September (zeros replace negative lower 95%CL).

^f Estimated number of *C. insularis* crossing an imaginary 1 km line perpendicular to the wind direction between 50–250 m above ground level.

^g Nightly hourly averages and 95%CL (over May-August; N = 2,706) [minimum temp].

Results and discussion

African aerial and ground surveillance

Overall, 25,431 insect specimens collected in West Africa on 308 panels (157 panels at 120-290 m agl, 84 panels at 1 m agl, and 67 control panels) were sorted and evaluated for the presence of Curtara insularis. Interception of C. insularis at altitude and at ground level occurred between May and August (Fig. 1) but not in September and October (sampling in other months were only performed in Mali). Although we cannot rule out high-altitude flights in the rest of the year, we consider the period between May and August as its migration period and unless otherwise stated, confined the summary statistics to this period (Table 1, Fig. 1). A total of 18 specimens of C. insularis were identified among samples collected in Agogo and Wenchi (Ghana) during May to August (9,113 insect specimens, Table 1), but none was found in Kenieroba (Mali) during these months (3,897 insect specimens, Table 1) or throughout the year (6,151 insect specimens). Most C. insularis (N = 14) were intercepted at altitude (160-190 m agl), 3 were collected at ground level, and 1 on a control panel (Table 1). A single specimen on the control panels (N = 45 control panels) as opposed to 14 specimens intercepted in these locations at altitude (N = 61 panels, Table 1) indicate that "contamination" near the ground (<50 m agl) is unlikely to account for the capture of so many specimens at altitude. At altitude, similar numbers of males (8) and females (6) were collected, indicating that both sexes equally engage in high-altitude flight unlike mosquitoes, in which females consist of > 80% from the aerial collection (Huestis et al. 2019; Yaro et al. 2022). All specimens were macropterous (having fully formed fore- and hindwings), albeit no polymorphism in wing development has been noted for this species.

The higher numbers of *C. insularis* at altitude and its relative larger fraction of the total insects on the panels among all specimens collected (Table 1) attest for its



Figure 1. Study area showing collection sites with wind patterns (during inferred migration season) and panel and aerial densities of *C. insularis* **A** map showing sampling sites in Ghana and Mali (balloon symbols) with the port of Abidjan, Ivory Coast (blue anchor). The arrow shows the predominant wind direction **B** nightly wind at altitude (300 m agl) by month during 2020-2021 at each sampling station (color) showing the direction (arrow) the insects will be carried towards, from the aerial sampling site (origin). The wind speed is indicated by the vector length (source: ERA5) **C** panel density of *C. insularis* at the different sampling stations (regardless of altitude) with corresponding color fringe (bottom) indicates sampling night used in this study. Blue shade indicates period of interception of *C. insularis* **D** aerial densities of *C. insularis* at Agogo and Wenchi at altitude (based on panels at 160-190 m agl). Fringe (bottom) indicates sampling dates at altitude in Ghana.

propensity to engage in high-altitude windborne dispersal. The scale of *C. insularis* movement at altitude was estimated as the average number of individuals flying between 50 and 250 m agl crossing a 1 km line perpendicular to the wind during a single night. With tens of thousands flying nightly at altitude across 1 km (Table 1), the expected number over the four-months season (Fig. 1) was in the millions, reflecting that this species had established robust populations in its source sites. Radar studies have shown that windborne insects fly nightly over swathes that are tens or even several hundreds of kilometers wide (Drake and Reynolds 2012; Hu et al. 2016). With a mean wind speed of 4.5 m/s, individual insects flying 2–8 hours would readily cover 30–150 km per night. High-altitude windborne dispersal has been reported in leafhoppers including a flight > 3,000 km over the

ocean (*Balclutha salturella* [Previously: *B. pauxilla*]), culminating in the invasion of Ascension Island (Ghauri 1983), and seasonal migrations over hundreds of kilometers in North America (*Empoasca fabae*, *Macrosteles quadrilineatus*, and *Circulifer tenellus*), and Asia (*Nilparvata lugens* and *Sogatella furcifera*) (Glick 1939; Taylor 1974; Carlson et al. 1992; Reynolds et al. 2017; Hu et al. 2019; Wu et al. 2019). In tropical West-Africa, temperature and humidity rarely restrict flight activity even at altitude (Table 1; Sanogo et al. 2021). Altogether, high-altitude dispersal of *C. insularis* is a potent strategy for spreading rapidly over large areas. These large number of migrants account for massive propagule pressure (Lockwood et al. 2005; Simberloff 2009) that could well explain the importance of this modality of dispersal compared with vehicular spread, which involves small number of specimens that often arrive in inhospitable spaces, e.g., storage facilities surrounded by urban environment, which limit reproduction success and establishment of populations.

Given the predominant wind directions in this region of West Africa (Fig. 1, Table 1), *C. insularis* is probably carried by the winds inland, encroaching up to at least ~300 km (Wenchi) from its supposed landing sites in one or more West Africa ports (Fig. 1). If it had been introduced through the largest port of West Africa, Abidjan, Ivory Coast, the predominant southwesterly winds would have carried it straight to the Agogo and Wenchi areas (Fig. 1), whereas Kenieroba is clearly off the predominant wind trajectories from the main Gulf of Guinea ports. Thus, invasion of locations such as Kenieroba may require over-land vehicular transport or considerably longer time using windborne dispersal. Whether *C. insularis* can establish populations in Kenieroba, Mali is an open question. Located within the Sudano-Guinean zone along the Niger river, the overall climatic conditions in Kenieroba are similar to those in Wenchi (Table 1) or other sites where *C. insularis* is found (below), albeit with a longer dry season (December-April). Other explanations in which *C. insularis* has arrived earlier at other region(s) of Africa cannot be rule out without additional data on its distribution on the continent.

Spread of Curtara insularis in the USA

The current distribution of C. insularis in the USA is based on 1,109 records (excluding 11 "in-transit" records) spanning the period of 2004–2023 (Suppl. material 1, 2: fig. S2). The records cover much of the southeastern and south-central USA (25°—36°N latitudes and 77°—102°W longitude, Suppl. material 2: fig. S2). There was minimal seasonal variation considering the month of observation and latitude or longitude (not shown), suggesting that C. insularis populations are perennial in that range. Given the number of the records, this range suggests a habitat-suitability space, which appears bounded to the north by annual temperature minima isotherm of -12 °C, described by vegetation zone 8 (USDA 2023) and to the west by annual precipitation isohyet of 510-630 mm (NOAA 2023). Despite their apparent correlation between these boundaries and the current limits on the C. insularis distribution, studies are needed to establish causal relationships of these hypothetical factors. Permissive combinations of temperatures and precipitation also occur along the Northwest coast (e.g., in California) and the Southwest (e.g., in Arizona). Indeed, C. insularis was intercepted in Florida on two trucks that originated from Arizona in 2022 and 2024 (FDACS-DPI records), although the species has not yet been reported from any western state. Future records of this species from
those territories may reveal the stability of the current range. We consider the range of records available up to December 2023 as the space in which range expansion could have occurred, evaluating how *C. insularis* "filled" it.

The interception of *C. insularis* in international ports (9 records since 2019) and on trucks entering Florida (3 records since 2004) provide evidence for the role of the maritime trade as well as vehicles overland in transporting this species. The port interceptions were in Florida (5), Houston Texas (2), Georgia (1), and Puerto Rico (1). These records substantiate that *C. insularis* can spread by all these means as well as by wind at altitude (above) as other invasive pests, such as *Helicoverpa* and *Spodoptera* moths (Jones et al. 2019). Given the large distances of transport by truck (daily average of ~1,000 km), airplane, or ships, the expected pattern of spread would be by long and irregular leaps in all directions (Suarez et al. 2001; Ahn et al. 2023; Lehmann et al. 2023), whereas windborne spread would predict a continuous incremental spread that resembles "diffusion process" that follows predominant winds.

The first record of C. insularis in the USA dates to January 2004 (Hillsborough County, Florida, Suppl. material 1). However, 42 specimens from 5 central-south Florida counties were identified that year, indicating the original introduction took place into that region earlier (Fig. 2A, Suppl. material 2: fig. S3). Over the next 5 years, it has spread throughout the state, reaching the panhandle by 2008, and expanding outside the state only in 2012 (Suppl. material 2: fig. S3). The relatively slow spread northward, given the numerous sightings in Florida and compared with subsequent years is incompatible with vehicular transport but can be attributed to "unfavorable" predominant winds which have a strong east-west component in relation to the narrow peninsula (~200 km wide), resulting in most windborne journeys ending at sea (Fig. 2A). That Florida is a major producer of most nursery stocks and diverse produce that are shipped by trucks efficiently all over the US, highlights the contrast between this mode of transport and the pattern of spread observed. In 2012, C. insularis was reported near Houston, Texas, and a year later near Lafayette, Louisiana (Suppl. material 2: fig. S3). With average nightly wind speeds 5.6 m/s (maximum of 16.9 m/s) we expect displacement ranges of 40-150 km/night over 2-8 hours of flight with up to 500 km during maximum recorded (nightly) windspeed. The closest C. insularis record was from Florida, > 900 km away from Houston (500 km away from Lafayette). The scarcity of westward winds from Florida and the large distance suggests that C. insularis was transported to Houston by a ship, truck, or airplane rather than by the wind (Fig. 2B, and below). Nonetheless, its arrival near Lafayette (Louisiana, Suppl. material 2: fig. S3) the following year is consistent with frequent eastward winds from Houston (Fig. 2B). In 2016, C. insularis was observed around Austin and Dallas (Texas) as well as near Columbus, Georgia (Suppl. material 2: fig. S3). Wind trajectories from Houston to Dallas were common and those towards Austin are modestly common, as are the trajectories from Tallassee to Columbus (Fig. 2B). The slower arrival into these destinations (~4 years) agrees with the modest frequency of corresponding wind trajectories. By 2017 and 2018, C. insularis was reported from South Carolina and in new counties of Texas, including a remote region of western Texas > 50 km from a major highway (Suppl. material 2: fig. S3). Fifteen of the new records were located consistent with wind trajectories from previously established populations (Fig. 2C), however 4 of 20 would require 9–11 hours windborne flight (at average nightly wind speed) and a single site near Matamoros, Mexico, would require ~16 hours flight or an



Figure 2. Maps showing range expansion of *Curtara insularis* in relation to projection of annual wind trajectories at altitude (300 m asl) from putative source(s) reported previously assuming 8-hour windborne flight and linear winds (broken lines) A expansion of *C. insularis* (2004—2011) with annual nightly projections of wind trajectories from Tampa 2005 (see text) B expansion of *C. insularis* (2012—2016) with annual nightly wind trajectories from Tallahassee and Houston 2012 (see text) C expansion of *C. insularis* (2017—2018) with projections of annual nightly wind trajectories from Jacksonville, Tallahassee, Columbus, Lafayette, Houston, Dallas, and Austin. Expansion of *C. insularis* (2018—2023) with projections of annual nightly wind trajectories from Florence, Columbia, Columbus, Crestview, Lafayette, Dallas, Austin, Matamoros (Mexico).

additional landing by ship at the nearby port of Brownsville Texas (Fig. 2C, Suppl. material 2: fig. S3). In 2019, C. insularis was reported from its northern-most point near Raleigh, North Carolina (~170 km from Florence, SC, Suppl. material 2: fig. S3), Tuscaloosa, Alabama (~260 km from Crestview FL), and Jackson, Mississippi (~130 km from Pensacola FL), as well as to other new localities, closer to previously established populations (Fig. 2D, Suppl. material 2: fig. S3). All these sites were consistent with common wind directions and distances from previously established populations (Fig. 2D, Suppl. material 2: fig. S3). Similarly, new counties in 2020 and 2021–2023 were also < 100 km from previously established sites and often from multiple such sites (Fig. 2D, Suppl. material 2: fig. S3), consistent with windborne spread. This approach is conservative because it assumes that the source populations of news localities are known, although unknown source sites may be closer to the new location, resulting in overestimating the distance these migrants actually passed. The average annual maximum distance of range expansion was 229 km (range: 0–950 km, Suppl. material 2: fig. S4). The arrival into Houston (recorded on 2012) presented an extreme outlier, indicating a different mode of range expansion from the rest (Fig. 2, Suppl. material 2: figs S3, S4). Excluding this

value, yields an average of annual maximum range expansion of 176 km (Suppl. material 2: fig. S4: inset). Both values appear considerably lower than the average maximum distances of truck transporting produce in the US.

Conclusion and implications

Our results show that C. insularis exploits multiple modes of long-range dispersal, including vehicular transportation on board of ships and trucks and windborne migration at altitude. Curtara insularis was first found in Africa by sampling at altitude. Based on its aerial density over Ghana, we estimate that annually, millions of C. insularis migrate at altitude across each 1 km sections perpendicular to the wind, representing a massive propagule pressure that probably exceeds by several orders of magnitude that of transport by vehicles. Extending these findings, we assess the relative importance of windborne migration compared with vehicular transport to the spread of C. insularis in the USA. Given the size of the habitat space this leafhopper has expanded to until 2023, it is notable that it took 5 years to reach the Florida panhandle from central Florida (~350 km), and 8 years to spread beyond Florida to other states. Likewise, all but three of the hundreds of records of inter-annual range-expansion exceed 300 km from the nearest previously known site. Had it regularly been transported by trucks (or airplanes) overland to the numerous new areas it was reported from, it would have spread from Central Florida and reach North Carolina (~1,000 km), or western Texas (~2,000 km) considerably faster than 19 or 15 years, respectively. Our data suggest that except 1-3 independent introductions to the US by the maritime trade (and possibly by trucks), C. insularis expansion overland has been incremental, diffusion-like process, which generally agrees with common wind trajectories. Thus, C. insularis range expansion in the US is better explained by high-altitude windborne dispersal following one or few successful journeys onboard ships. How unique is C. insularis among invasive insects in exploiting high-altitude windborne dispersal? Because strong dispersive capacity is a key trait of invasive species (Mack et al. 2000; Lounibos 2002; Hulme et al. 2008; Anderson et al. 2010; Renault et al. 2018; Hu et al. 2019; Jones et al. 2019) and because of the diverse insect faunas at altitude (Chapman et al. 2004; Drake and Reynolds 2012; Hu et al. 2016; Florio et al. 2020; Yaro et al. 2022; Huang et al. 2024), we propose that-high altitude windborne dispersal may well be especially common among invasive insect species. Some of these species pose severe risks to biodiversity, food security, and public health (above), and are exacerbated by anthropogenic changes including climate change (Cao and Feng 2024). Therefore, aerial surveillance 10-30 km downwind from major ports might complement traditional surveillance procedures to discover invading insect species early and predict their likely destinations-information that can improve elimination efforts before they spread over large areas—when elimination may be an especially viable option.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

The study was conceived by TL and RN and the study design was shaped with inputs from JWC, DR, SEH, SH, YA, MB, LC, CB, RB, and KOD. Field work, data management, and initial specimen processing were carried out by AD, ASY, YA, ARM, CMO-A, GA-B, CMP-Z, MD, ZLS, and SD. Laboratory analysis was done by RN, and LC, data compilation from different sources were carried out by SEH, SH, CEN, MB, TL and RN. Data analysis was carried out by TL with inputs from MB, LC, RN, RB, DRR, JWC, SEH. The first draft was written by TL and RN and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Types=&species=&vectorSpecies=&surveyTypes=&deletionType=HRP2_PROPORTION_DE-LETION&plasmodiumSpecies=P_FALCIPARUM&drug=DRUG_AL&mmType=1&exclude-LowerPatients=false&excludeLowerSamples=false&endemicity=false&countryMode=false&storyMode=false&storyModeStep=0&filterOpen=true&filtersMode=filters&years=1985%2C2023 [accessed March 21, 2024.2024]

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Supplementary material 1

The sighting records of C. insularis in the USA

Authors: Rita Nartey, Lourdes Chamorro, Matt Buffington, Yaw A. Afrane, Abdul Rahim Mohammed, Christopher M. Owusu-Asenso, Gabriel Akosah-Brempong, Cosmos M. Pambit-Zong, Solomon V. Hendrix, Adama Dao, Alpha S. Yaro, Moussa Diallo, Zana L. Sanogo, Samake Djibril, Susan E. Halbert, Roland Bamou, Catherine E. Nance, Charles R. Bartlett, Don R. Reynolds, Jason W. Chapman, Kwasi Obiri-Danso, Tovi Lehmann

Data type: xlsx

- Explanation note: As described in the Methods, this compilation includes publicly available records of observations of *C. insularis* from BugGuide.net (n = 22; (BugGuide 2024)), iNaturalist (n = 827; (iNaturalist 2024)) that met "Research grade" standard (verified by SVH) and records available from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS-DPI) (N = 266). Interception records in trucks or in ports were excluded as they may not represent populations (see Methods for details).
- Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/neobiota.96.130615.suppl1

Supplementary material 2

Compilation of distribution sighting records of Curtara insularis in the USA

Authors: Rita Nartey, Lourdes Chamorro, Matt Buffington, Yaw A. Afrane, Abdul Rahim Mohammed, Christopher M. Owusu-Asenso, Gabriel Akosah-Brempong, Cosmos M. Pambit-Zong, Solomon V. Hendrix, Adama Dao, Alpha S. Yaro, Moussa Diallo, Zana L. Sanogo, Samake Djibril, Susan E. Halbert, Roland Bamou, Catherine E. Nance, Charles R. Bartlett, Don R. Reynolds, Jason W. Chapman, Kwasi Obiri-Danso, Tovi Lehmann

Data type: docx

- Explanation note: **fig. S1.** *Curtara insularis*: lateral view showing reddish ring spots on wings (source: Solomon V. Hendrix). **fig. S2.** Map showing distribution of *C. insularis* in the USA based on the records compiled in this paper (Suppl. material 1) by four-year intervals. Blue line is schematically drawn to represent annual temperature minima isotherm of -12 °C (USDA vegetation zone 8 (USDA 2023) and the black line was drawn to represent the annual precipitation isohyet of 510–630 mm (NOAA 2023). **fig. S3.** Annual range expansion of *C. insularis* showing each year new records (red) and cumulative previous records (green). Range expansion is defined as location outside the polygon connecting all extreme sites (Methods). Map dimension changes based on sighting locations and years with minimal range expansion are shown as inset (2014) or reduced in size (2022–3). **fig. S4.** Over time variation in the maximum annual range-expansion distance measured between each year new sightings and the closest previous sightings. Red circle around the extreme outlier (2012) shows a distance of 950 km from the nearest previous record. Inset: The same graph excluding the 2012 extreme outlier. Arrows separate periods of apparent larger annual expansion distances.
- Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

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Research Article

Supporting early detection of biological invasions through short-term spatial forecasts of detectability

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Abstract

Early detection of invasive species is crucial to prevent biological invasions. To increase the success of detection efforts, it is often essential to know when key phenological stages of invasive species are reached. This includes knowing, for example, when invasive insect species are in their adult phase, invasive plants are flowering or invasive mammals have finished their hibernation. Unfortunately, this kind of information is often unavailable or is provided at very coarse temporal and spatial resolutions. On the other hand, opportunistic records of the location and timing of observations of these stages are increasingly available from biodiversity data repositories. Here, we demonstrate how to apply these data for predicting the timing of phenological stages of invasive species. The predictions are made across Europe, at a daily temporal resolution, including in near real time and for multiple days ahead. We apply this to phenological stages of relevance for the detection of four well-known invasive species: the freshwater jellyfish, the geranium bronze butterfly, the floating primrose-willow and the garden lupine. Our approach uses machine-learning and statistical-based algorithms to identify the set of temporal environmental conditions (e.g. temperature values and trends, precipitation, snow depth and wind speed) associated with the observation of each phenological stage, while accounting for spatial and temporal biases in recording effort. Correlation between predictions from models and the actual timing of observations often exceeded values of 0.9. However, some inter-taxa variation occurred, with models using direct predictors of phenological drivers and trained on thousands of observation records outperforming those relying on indirect predictors and only a few hundred training records. The analysis of daily predictions also allowed mapping European-wide regions with similar phenological dynamics (i.e. 'phenoregions'). Our results underscore the significant potential of opportunistic biodiversity observation data in developing models capable of predicting and forecasting species phenological stages across broad spatial extents. By enhancing our current ability to anticipate the phenological stages of invasive species, this approach has the potential to significantly improve decision-making in invasion surveillance and monitoring activities.

Key words: Citizen science, early warning systems, field surveying, invasion monitoring, phenology tracking, real-time forecasting



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Introduction

Invasive alien species are a major environmental problem, severely impacting biodiversity, economies and public health (IPBES 2023). As human activities continue to transport and introduce alien organisms outside native regions (Hulme 2021; Capinha et al. 2023), the number of new invasions is expected to grow (Seebens et al. 2021), increasing the magnitude of their impacts (Bacher et al. 2023). As a result of this, successful invasion prevention efforts are key for safeguarding biodiversity, agriculture, economic development and human well-being (Vilà and Hulme 2017; IPBES 2023). In this regard, it is particularly important to detect non-native species in the early stages of the invasion process, as it significantly improves the effectiveness of control measures (Tobin et al. 2014; Larson et al. 2020; Martinez et al. 2020).

Current efforts in the surveillance and early detection of alien species encompass a large diversity of approaches, including camera and chemical traps, eDNA analysis, remote sensing and visual surveys conducted by experts and citizen scientists (Larson et al. 2020). Each of these approaches has distinct strengths and limitations and optimal outcomes are likely achieved through the integration and assimilation of their collective data (Larson et al. 2020; Fricke and Olden 2023). A key factor in improving the success and cost-effectiveness of many of these methods is understanding when invasive species are in phenological stages that maximise detection efficiency. For instance, remote sensing often targets periods of higher species conspicuity, such as plant flowering or increased greenness, to enhance detection accuracy (e.g. Müllerová et al. (2017); Wijesingha et al. (2020)). Similarly, trap-based surveillance programmes are often tailored to specific life stages of the target species and the deployment of these traps aims to coincide with the expected timing of these stages (Takeuchi et al. 2023; Nguyen et al. 2024). Likewise, visual-based field surveys - either performed by experts or citizen scientists - also greatly benefit from knowing when phenological stages of higher visual conspicuousness are reached (e.g. the adult phase of an invasive insect species or the blooming of an invasive plant), sustaining the development of surveys and monitoring activities when detectability is higher (Pocock et al. 2023).

Despite the importance of understanding the optimal timing for surveillance and early detection, information on species detectability levels is often unavailable, inadequate or of limited value. For most invasive species, including highly problematic ones, the available information on these levels typically consists of dates of relevant life cycle stages observed in other regions (e.g. EFSA et al. 2020) or the months or seasons when these stages are typically observed (Veenvliet et al. 2019). However, this type of information can overlook significant inter-regional and inter-annual differences resulting from the natural variation of drivers of phenology, such as temperature and precipitation (Godoy et al. 2009). Some exceptions exist for species for which phenological models have been developed. These models, whether process-based or data-driven, have yielded successful predictions of phenology (e.g. Barker et al. (2020); Reznik et al. (2022)), thereby supporting invasion surveillance efforts (e.g. Taylor et al. (2020); Takeuchi et al. (2023); Barker and Coop (2024)). However, applying these models to a large number of invasive species — i.e. taxonomic scalability — can present significant difficulties. Process-based models require species-specific eco-physiological parameters, which can be unavailable or hard to obtain for the species of interest (Chuine and Régnière

2017; but see Barker et al. (2020) and Takeuchi et al. (2023)). Conversely, most data-driven phenological modelling approaches rely on long-term observational data, such as phenological time series (e.g. Taylor and White (2020)), which are typically limited in spatial and taxonomic coverage (Park et al. 2021). These limitations can hinder the application of such approaches to a broad range of taxa.

Recently, we have demonstrated how temporally and spatially discrete biodiversity observation data, widely available from popular online repositories, such as the Global Biodiversity Information Facility (GBIF: https://www.gbif.org/) or iNaturalist (https://www.inaturalist.org/), can be used to estimate the timing of ecological phenomena across regions (Capinha et al. 2024). This approach is based on the concept of the phenological niche (Post 2019) and, in simple terms, involves using these data to represent the set of temporal environmental conditions under which an ecological phenomenon of interest (such as a species' phenological stage) occurs. From a practical standpoint, this can be achieved by applying statistical or machine-learning models to identify the 'envelope' of temporal environmental conditions associated with the observation of the phenomenon of interest. Once calibrated to perform this identification, the models can then be coupled with environmental predictor data (such as spatial time series of meteorological variables) and used to predict the probability of the phenomenon occurring over time (e.g. each day) and across regions.

Our previous work (Capinha et al. 2024) focused on demonstrating the conceptual and applied feasibility of this approach. Here, we aim to specifically highlight its potential for supporting efforts of early detection and monitoring of invasive species. This is done by demonstrating its use for predicting the timing of phenological stages of relevance for field surveying, at a daily resolution and for several days in advance, across Europe. We examine four well-known invasive species in Europe, offering different levels of observation data availability, and explore model accuracy, uncertainty and the regional patterns of predicted phenological dynamics. Ultimately, we demonstrate that this approach has significant potential to provide information for invasion surveillance efforts, while also offering relevant taxonomic scalability.

Methods

Collation of observation record data

We focus on four alien species that are established in Europe: the freshwater jellyfish (*Craspedacusta sowerbii*), the geranium bronze butterfly (*Cacyreus marshalli*), the floating primrose-willow (*Ludwigia peploides*) and the garden lupine (*Lupinus polyphyllus*). The levels of visual detectability for these species change considerably throughout the year. The freshwater jellyfish is presumed to be native to regions of Asia and has been introduced in most continents of the world. However, its alien distribution remains poorly known, largely because the most visible part of its life cycle involves small medusae that appear for only a few months each year (Marchessaux et al. 2021). The geranium bronze is a small butterfly native to southern Africa and currently invading parts of central and southern Europe. Like most insects, its adult (butterfly) stage has higher visibility due to increased mobility and conspicuous colours. The floating primrose-willow is an aquatic plant native to Oceania and the Americas, with invasive populations in countries of central and southern Europe. This species produces bright yellow flowers, which facilitate its identification amongst surrounding vegetation (Booy et al. 2015). Finally, the garden lupine is a plant native to western North America that is now widespread in many temperate regions of the world, including central and northern Europe. This species produces prominent flower spikes (often violet, but sometimes also pink or white) that greatly facilitate its detection, including via remote sensing (Wijesingha et al. 2020).

Following our previously described framework (Capinha et al. 2024), we collected observation records supported by photographs for each of these species from GBIF. These records were sourced globally (i.e. not limited to Europe) to maximise the range of environmental conditions sampled. To ensure temporal consistency with the environmental predictors (see below), we used georeferenced records from a 7-year period between 2016 and 2022 that included the complete date of observation (day, month and year). GBIF is a major online repository of biodiversity observation data, including from well-known and highly participated citizen-science projects (e.g. iNaturalist.org and observation.org), which typically provide supporting visual media. As the number of records obtained from GBIF for the freshwater jellyfish was low, we also searched for observation records of this species in the USGS Non-indigenous Aquatic Species portal (https://nas.er.usgs.gov) and a few additional sources (see link for full list in the Data Resources section).

We visually checked the photographs supporting each observation record of the four species and kept only those that clearly showed the medusa stage of the freshwater jellyfish, the butterfly stage of the geranium bronze and the flowering stages of both the floating primrose-willow and the garden lupine. Records with images suggesting that the specimens were under human-care (e.g. garden lupine in places showing garden-like features) were excluded. Likewise, we also excluded GBIF records where the observation date was the first day of the month and the observation time was '00:00:00'. These are typically records where only the month and year of observation are known and the first day of the month is assigned by default, i.e. the full date of the record may not be precise (Belitz et al. 2023). In total, we obtained 240 records for freshwater jellyfish medusae, 3,879 for geranium bronze butterflies and 1,688 and 10,345 records for floating primrose-willow and garden lupine flowers, respectively (Suppl. material 1: fig. S1).

Environmental drivers

We collected a time series of global-scale maps representing daily conditions of maximum, minimum and mean temperature, accumulated precipitation, wind speed and accumulated snow. These factors are expected to be drivers of the timing of occurrence of the species life stages of interest, according to previous research (Favilli and Manganelli 2006; Ludewig et al. 2022; Marchessaux et al. 2022). The data were collected from the Global Forecast System (GFS), a large-scale NOAA weather forecast model (https://www.ncei.noaa.gov), covering the period between 15 January 2015 (the first day the data is available) to 31 December 2022. GFS data are provided at 0.25° spatial resolution, for multiple hourly intervals and for each model run that takes place at 00, 06, 12 and 18 UTC daily. The daily-resolution maps were obtained by aggregating the first six-hour values provided by each model run. This means we used the forecast for 00:00–06:00, 06:00–12:00, 12:00–18:00 and 18:00– 00:00 UTC, covering the full 24 hours of each day. The temporal immediacy of this period in relation to the timing of model runs results in highly accurate weather forecasts (NOAA 2022). Models employing these data achieve results comparable to those using climate re-analysis data - traditionally for ecological forecasting - like ERA5 (Capinha et al. 2024). Crucially, NOAA also offers real-time access to GFS data, including weather forecasts extending several days into the future, meeting our objective to deliver predictions in real-time and for short-term forecasting.

Spatial bias removal

We implemented a set of procedures to minimise potential spatial and temporal biases in the observation data. Spatial bias refers to unequal numbers of records in distinct regions, which can lead to model responses being 'dominated' by the patterns occurring in oversampled regions. Temporal observation bias arises from unequal levels of recording effort within and across years, confounding the actual temporal signal of phenological events.

To address these biases, we followed the procedures we proposed earlier (Capinha et al. 2024). Specifically, and to minimise spatial bias, we kept only one record located in the same grid cell and having the same date of observation. Next, we downsampled observations in oversampled regions. For this purpose, we used a reference grid of 250 km × 250 km squares, for which we identified squares that were upper outliers in terms of record count (i.e. n > third quartile + 1.5 * interquartile range). For these areas, we randomly subsampled a number of records equal to the outlier threshold.

Temporal bias removal

Our framework includes an optional procedure to minimise temporal bias, named 'benchmark taxa approach' (Capinha et al. 2024). This procedure uses taxa that maintain a consistent visual appearance throughout the year as indicators of variations in recorder activity (e.g. citizen scientists). We use pine species for this purpose because they are evergreen and have a fairly consistent appearance year-round. Since these taxa do not undergo significant seasonal changes, temporal variations in their recording frequency are likely due to fluctuations in recording effort rather than changes in the taxa themselves. In other words, we expect that the temporal patterns in recordings of these species serve as a proxy for general citizen-science activity levels (see further description of the rationale in Capinha et al. (2024)).

The temporal variation in the frequency of records for these taxa is related to variables expected to mediate levels of recording effort (e.g. days of the week, months of the year and weather conditions) by means of a statistical model such as a generalised linear model. Based on the relationships identified, the temporal biases in records of the phenomenon of interest can be minimised by a subsampling procedure, where records made in periods of higher levels of recording intensity receive a lower probability of being selected for model development. We demonstrated this approach previously and its application delivered similar performance to the models without using it. However, it is not clear if this outcome can be expected in the generality of phenomena. We therefore performed all the analyses using event observation data with this correction (described in Suppl. material: text S1) and without it. The results were similar in both approaches (see Results); therefore, the approach using the temporally corrected data is presented only in the Suppl. material 1.

Environmental characterisation of records

We next characterised the meteorological conditions preceding each event record. We used a total of 67 features representing multiple features of temperature (e.g. maximum, minimum and mean values, growing degree days and cold accumulation), accumulated precipitation, accumulated snow and mean wind speed for distinct preceding periods ranging from days, weeks and months up to a year (see full list in Suppl. material 1: table S1).

Additionally, we assembled a second set of data aimed at representing the meteorological conditions that are generally available in the location of each of the events (i.e. the background environmental conditions). This was performed using 'temporal pseudo-absences' (Capinha et al. 2024), which correspond to records having the same geographical coordinates as event records, but with dates drawn at random within the temporal range of the event data. A total of 12 pseudo-absence records were generated from each event record and each was characterised using the same set of 67 environmental features. The number of pseudo-absence records per observation was determined subjectively, based on preliminary assessments, which indicated a good balance between the diversity of sampled conditions and the volume of data produced.

Model training and evaluation

Prior to model fitting, we tested for the presence of multicollinearity amongst the predictors. For this purpose, we measured their variance inflation factor ('VIF') and excluded any predictor with a VIF value above 10 (Gareth et al. 2021). We then trained three machine-learning algorithms: random forests (RF), boosted regression trees (BRT) and generalised linear models with lasso regularisation (GLM-lasso), in distinguishing the conditions associated with the phenological stages of interest and those represented by the temporal pseudo-absences. These algorithms were selected because they are commonly used for ecological modelling and prediction and often rank amongst the best performing, including when transferred to new spatial settings (Zhang et al. 2019; Valavi et al. 2023).

The implementation of these models was performed in R (R Core Team 2024), using the 'randomForest' package for RF (Liaw and Wiener 2002), the 'dismo' package for BRT (Hijmans et al. 2017) and 'glmnet' for GLM-lasso (Hastie et al. 2021). We optimised several parameters within these functions to improve model fitting. Random forests used 2,000 individual trees (instead of the default 500) to increase the chances of the relatively large number of predictor variables and samples being adequately represented in the final ensemble. For the BRT models, the number of trees in each ensemble was automatically determined by the `gbm. step' function of the 'dismo' package, with a tree complexity of 3 (allowing for interactions amongst predictor variables) and a learning rate of 0.005. Additionally, for all three algorithms, we addressed the class imbalance in the data (i.e. one event observation record for every 12 pseudo-absence records), which could skew models towards over-predicting the dominant class (pseudo-absences). For GLM-lasso and BRTs, this was made through a weighting parameter ('weights' for GLM-lasso and `site.weights` for BRTs), assigning class-proportional weights to each sample. For Random Forests, the adjustment was made using the 'sampsize' parameter of the 'randomForest' function, ensuring that each individual tree used an equal number of event observation records and temporal pseudo-absences.

To evaluate the predictive performance of the models, we used a leave-oneyear-out cross-validation procedure. This involved excluding the data from one year for model calibration and using it to assess the predictive ability of models trained on the remaining years. The procedure was iterated so that the data from each year served as an evaluation set. To measure the models' performance, we used the Boyce Index, initially proposed for species distribution models (Hirzel et al. 2006). The Boyce Index measures the correlation between the frequency of observation records and predicted probability values. In the context of our work, a strong positive correlation implies that event observations are concentrated in periods predicted with higher probability values, whereas correlations near zero indicate predictions akin to those expected from a model that assigns probability values randomly throughout the year. We preferred this metric over discrimination-based metrics, like the commonly-used area under the receiver operating characteristic curve (Pontius and Parmentier 2014), because these latter metrics would involve assessing the misclassification of temporal pseudo-absences. However, in the context of our work, these pseudo-absences capture the environmental variation that is available, including periods that are potentially suitable for the occurrence of our focal phenological stages. This becomes particularly critical for models of phenological stages with extended seasons of occurrence, where temporal pseudo-absences during suitable periods are more likely. Consequently, this would likely result in an underestimation of the models' true predictive performance due to the misclassification of pseudo-absences.

We performed the Boyce Index calculations using the *ecospat.boyce* function from the *ecospat* R package (Broennimann et al. 2015), employing the Pearson correlation coefficient as a metric. The measurements were performed for the three algorithms and for an ensemble of these predictions corresponding to their average.

Real-time, days-ahead forecasts of event probability

The ability to predict species' phenological stages several days in advance can guide decision-making on the optimal timing of early detection efforts for invasive species (Barker et al. 2020; Crimmins et al. 2020; Barker and Coop 2024). Consequently, an important objective of our work was to use the models developed to provide real-time phenological forecasts for several days into the future. We performed this, based on GFS weather forecasts and for up to 9 days into the future, a forecast horizon that is broad enough to guide short-term surveying decisions, but which is not too close to the limit of the GFS forecast horizon (16 days), allowing to accommodate technical challenges such as server downtime and decreased temporal resolution of GFS data after 10 days. These forecasts were implemented in real-time, providing short-term forecasts of detectability and can be accessed at https://www.natureforecast.org.

An important question is whether the forecasts lose accuracy as they extend further into the future and, if so, to what extent. To address this, we calculated the Boyce Index for phenology forecasts derived from GFS weather data produced immediately before the target day (i.e. the 18:00 UTC run of the day before). We then compared these with forecasts based on weather data generated 3, 6 and 9 days in advance. This assessment covered a 10-month period from 1 June 2023 to 31 March 2024, corresponding to the timeline from the real-time deployment of the forecasting models to the writing of this work. As evaluation data, we gathered observation records for this period from GBIF, keeping only those that represented the life stages of interest and performing the same initial data-cleaning procedures as for calibration data (i.e. removing records without full date attributes and duplicates in space and time). Only records in Europe were considered, matching the geographical focus of the work (i.e. where the four species are invasive). Observation records for *L. polyphyllus* for this period were highly voluminous (> 19,000 records). To reduce the time resources needed to visually identify the life stage of each observation, a subset of 1000 randomly selected records was considered for processing.

Mapping phenoregions

Identifying regions with similar year-to-year phenological patterns ("phenoregions"; White et al. (2005)) allows defining areas where surveillance programmes for invasive species could be implemented in the same periods. To identify these regions, we used the phenology predictions from RF models at 5-day intervals for the 7-years period (from 2016 to 2022), across Europe. We used the predictions from this algorithm because it consistently performed well, ranking as the best or second-best across all species (see Results). To minimise the risk of extrapolation errors, i.e. making predictions for conditions far from those represented in the training data, we only made predictions for regions with Köppen climate classes (from Beck et al. (2018)) where each phenological stage had at least five observation records. Although simple, this method allows us to discriminate between regions with higher or lower propensities for model extrapolation. While this approach was effective for our purposes, future research could benefit from incorporating finer-scale assessments of extrapolation.

We applied a k-means algorithm to cluster regions based on the temporal variation in predicted values, using the 'elbow' method to determine the optimal number of clusters (Syakur et al. 2018). For each identified region, we then calculated the mean and standard deviation of inter-annual variation.

Results

Predictive performances

Overall, the predictions from models (Fig. 1, Suppl. material 1: figs S2, S3) achieved high performances. The predictions of imago-stage of the Geranium bronze attained a cross-model median correlation with the timing of observations of 0.95. GLM-Lasso achieved the highest median performance (r = 0.97), followed by RF (r = 0.96), multi-model ensemble (r = 0.95) and BRT (r = 0.94) (Fig. 2). Pairwise Kruskal-Wallis tests indicate non-significant differences in these performances (i.e. $p \ge 0.05$; Suppl. material 1: table S2). Likewise, the flowering timings of the floating primrose-willow and the garden lupine were also well captured by the models, with median cross-model correlations of 0.94 and 0.91, respectively. For the floating primrose-willow, the multi-model ensembles, RF and GLM-Lasso models had the best performance (median r = 0.94), with BRT performing slightly the worst (median r = 0.88). However, as before, these differences were not statistically significant (Kruskal-Wallis $p \ge 0.05$; Suppl. material 1: table S2). In contrast, for the garden lupine, RF displayed a statistically significant superiority (median r = 0.96; Kruskal-Wallis $p \le 0.05$; Suppl. material 1: table S2), over remaining algorithms. BRT was second best (median r = 0.92),



Figure 1. Examples of daily predictions obtained from the modelling approach. These represent the probability of occurrence for each modelled phenological stage for four species (imago-stage of the Geranium bronze [**a**]; medusae of the freshwater jellyfish [**b**], flowering of the floating primrose-willow [**c**] and flowering of garden lupine [**d**]), for 1 July 2023. Predictions were obtained using random forests, the best performing algorithm, trained with observational data corrected for spatial bias.

followed by multi-model ensemble and GLM-Lasso (median *r* for both = 0.88), but with no statistically significant differences amongst these (Kruskal-Wallis $p \ge 0.05$; Suppl. material 1: table S2). Predictive performance for the medusae stage of the freshwater jellyfish was the lowest, but still reasonable – with a median correlation of 0.73 across models (Fig. 2). Predictions from the ensemble approach demonstrated a substantially higher performance (median r = 0.82), followed by RF (median r = 0.79), GLM-Lasso (r = 0.64) and BRT (r = 0.42), but these differences were also not statistically significant (Suppl. material 1: table S2).

Relevantly, models trained with data addressing both spatial and temporal biases showed similar predictive performances as those addressing only spatial biases (Suppl. material 1: fig. S4). Specifically, the predictions of imago-stage of the Geranium bronze retained the highest cross-model median correlation with the timing of observations (0.95), followed by the garden lupine (0.91), the floating primrose-willow (0.90) and the freshwater jellyfish (0.67). Performances for the Geranium bronze were highest for GLM-Lasso, RF and multi-model ensemble (median r = 0.95), followed by BRT (median r = 0.90). For the floating primrose-willow, the best predictions were achieved by multi-model ensembles and RF (median r = 0.91), followed by GLM-lasso (median r = 0.90) and BRT (median r = 0.87). For the garden lupine, the best performances were achieved by RF



Figure 2. Results of Boyce Index corresponding to Pearson correlation values between predicted probabilities of event occurrence and the frequency of event observation records of the imago stage of the Geranium bronze butterfly (\mathbf{a}), medusae of the freshwater jellyfish (\mathbf{b}), the flowering of floating primrose-willow (\mathbf{c}) and the garden lupine (\mathbf{d}). The boxplots represent the variation of correlation values assessed for 7 years (2016 to 2022), using three modelling algorithms (boosted regression trees, BRT; generalised linear models with lasso regularisation, GLM-Lasso; random forest, RF) and an ensemble of previous algorithms (Ensemble), trained with observation data corrected for spatial bias.

(median r = 0.97), followed by multi-model ensembles and BRT (median r = 0.90) and by GLM-Lasso (median r = 0.88). Finally, for the freshwater jellyfish, the best performances were achieved by RF and GLM-Lasso (median r = 0.68), followed by multi-model ensembles (median r = 0.66) and BRT (median r = 0.44). As for models using observation data corrected for spatial bias only, the only statistically significant difference amongst algorithms was verified for the garden lupine, with RF significantly outperforming remaining ones (Suppl. material 1: table S2).

We also assessed the performance of days-ahead forecasts across Europe over a 10-month period (Fig. 3; Suppl. material 1: fig. S5). For the freshwater jellyfish and the floating primrose-willow, the observation records used for model evaluation during this period were relatively limited (25 and 42, respectively), while they were more abundant for the Geranium bronze (1,187) and the garden lupine (126 records from a random sample of 1,000 processed records). Overall, the forecasts demonstrated a high correlation with the timing of observed life stages modelled, with values generally close to 0.8 or higher (Fig. 3; Suppl. material 1: fig. S5). Additionally, there was no apparent pattern of decreased model performance as the forecast horizon extended (e.g. from 1 day ahead to 9 days ahead).

Regions of similar phenology ('phenoregions') and associated temporal patterns.

Using the predictions of the random forest algorithm trained with observation data corrected for spatial bias, we identified five phenoregions for the Geranium bronze butterfly in Europe (Fig. 4a), with the peak occurrence ranging from early June to late



Figure 3. Boyce Index values for forecasts made 1, 3, 6 and 9 days in advance. These values correspond to the Pearson correlation coefficient between predicted probabilities of event occurrence from July 2023 to March 2024 and the frequency of event observations recorded during the same period. The values are reported for three modelling algorithms—boosted regression trees (BRT), generalised linear models with lasso regularisation (Lasso) and random forest (RF)—as well as an ensemble of these algorithms (Ensemble), all trained with observation data corrected for spatial bias.

November in the southernmost region (Fig. 4b). The remaining regions have peak occurrences between August and September, with northern latitudes having a shorter season of occurrence and the opposite being true for southern latitudes. Recorded observations of butterflies of this species are concentrated in the summer (Fig. 4c), especially in the mid- to higher latitude regions, in agreement with the predictions.

Predictions of the timing of occurrence of medusae of the freshwater jellyfish were clustered into four phenoregions, peaking between late August and September. However, southern regions exhibit higher probabilities of occurrence over substantially longer periods and, conversely, shorter periods are predicted for northern regions. Medusae observations take place in the months of predicted peaks, except for the mid-latitude region covering most of Central Europe, where they concentrate in September and November — a period when the predicted probabilities are already declining.

For the floating primrose-willow, four regions were identified (Fig. 4g), with the southern regions showing earlier and more extended flowering periods (Fig. 4h). The timing of observational data matches well with the predictions for the region including most of Mediterranean Europe, although very few records are available for the other regions (Fig. 4i).

Predicted timings of flowering for the garden lupine were classified into four phenoregions (Fig. 4j), with probabilities peaking from early May to late June and occurring earlier in southern regions (Fig. 4k). Observational data also largely agree with the predicted patterns, especially for the two northernmost regions, which have most records (Fig. 4l).

Importantly, identified phenoregions and associated temporal patterns were strikingly similar to those obtained, based on models calibrated with temporally unbiased observation data (Suppl. material 1: fig. S6). This further suggests the robustness of predictions to temporally varying levels of recording effort.



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Discussion

Efforts for the early detection of biological invasions greatly benefit from understanding when and where invasive species enter life cycle stages that enhance detectability (Müllerová et al. 2017; Barker and Coop 2024). Here, we have demonstrated that temporally discrete biodiversity observation data, as made widely available in well-known public repositories such as GBIF and contributed by citizen science (Bonney 2021; Heberling et al. 2021), can be used to estimate the timing of occurrence of such stages, including across broad spatial regions (e.g. continents) and at very high temporal resolution (e.g. daily).

Our approach was demonstrated by modelling a distinct life stage for each of four species. The results obtained demonstrate a varying ability of trained models to identify the temporal environmental variation associated with observing the life stages of interest. For three of the life stages modelled, the agreement between predictions and the timing of observation was, across models, very high, with median correlations regularly above 0.9. However, for one of them (the medusae stage of the freshwater jellyfish), the performance was lower (though still high; cross-model correlations = 0.73 and 0.67). This lower performance could be partly attributed to the use of terrestrial predictors (e.g. air temperature, wind speed and snow cover), which serve as indirect proxies for aquatic conditions and limit the ability of models to capture phenological drivers with high precision. For aquatic species, variables such as water temperature and resources are likely critical drivers of phenology (Thackeray et al. 2016; Woods et al. 2022), but were not included in our models due to a lack of data meeting the spatial extent and temporal resolution required. Therefore, while our approach demonstrates that good predictive performances can be achieved, incorporating predictors that accurately represent the primary drivers of phenology will likely be crucial.

The lower performance observed for medusae of the freshwater jellyfish also coincides with the lowest number of observation records available (few hundreds), significantly fewer than those available for the remaining life stages (in the order of thousands). This also suggests that, as with the generality of modelled phenomena, the size of the training data can be a limiting feature. Indeed, the predictions allowed by our approach, which can be made daily and over wide geographical areas, involve a high dimensionality of conditions in the prediction space resulting from the multiple states of preceding conditions for each environmental variable and their joint combination. Hence, it is a reasonable expectation that the calibration data should be necessarily large in number to represent this variability; otherwise, extrapolation may occur and the uncertainty of predictions will be higher and possibly also less accurate (Yates et al. 2018).

In this work, we did not explicitly quantify extrapolation, as it presents significant challenges in models that deal simultaneously with spatial and temporal variation. Properly assessing extrapolation in this context requires considering both its magnitude — ideally weighted by the relative importance of each predictor in the models — and its temporal recurrence. However, commonly used methods like Mahalanobis distance (Mesgaran et al. 2014) and mobility orientated-parity (Owens et al. 2013) do not adequately address these complexities. Due to these limitations, we chose not to perform a detailed extrapolation analysis, acknowledging the need for more advanced techniques in future research. Despite this, it is very positive to verify that sample sizes on the order of magnitude of a few thousands (as available for the remaining species), consistently deliver very good predictions. Indeed, the number of

invasive species for which life stages of relevance for early detection and monitoring are represented by similar amounts of records can be presumed to easily reach several thousands, considering the enormous volumes of biodiversity data becoming available each year, particularly through citizen science (Bonney 2021; Heberling et al. 2021). This highlights the potential taxonomic scalability of our approach.

It is well-known that multi-sourced opportunistic biodiversity observation data, as used to train our models, can suffer from substantial spatial and temporal bias, often hindering efforts of use for prediction (Isaac and Pocock 2015; Brown and Williams 2019). We applied a set of procedures to minimise the effect of these biases. Specifically, we corrected for the temporal bias component (see also Capinha et al. (2024)). However, we also found that models having this correction and those without it are largely equivalent in terms of predictions. This was also verified in our previous work demonstrating the general modelling workflow (Capinha et al. 2024), where we interpreted these results by virtue of the way models are trained. Conceptually, our models contrast two point clouds in the environmental space, one representing the conditions associated with the occurrence of the life stage of interest and the other the generality of conditions available in the regions of its occurrence. As random records ('pseudo-absences') are generated at a fixed ratio to observation records, observations from periods with lower recording effort will have a similar relative influence as those from periods with higher recording effort. This does not mean that predictions made for the least represented conditions will be as accurate as those made for more populated spaces of the environmental space. Rather, it means that having more populated spaces of the environmental space will not direct the models' inference towards these conditions.

Our approach is also capable of forecasting the probability of occurrence of the life stages assessed, in real time and for several days into the future. This capacity is perhaps the most impactful aspect of our work for practical applications. Providing these forecasts daily and across extensive areas (such as the European continent in this case) could support a variety of decision-making processes related to the timing and efficacy of invasive species detection efforts. Of relevance, we also observed that the performance of forecasts remains largely stable over the forecast horizon considered. This consistency may result from the relatively brief time span considered (nine days) and the inherent temporal correlation amongst phenological events, which tend to unfold gradually and slowly over time (considering the daily temporal resolution used).

The identification of phenoregions (i.e. regions sharing similar phenological dynamics), as allowed through the spatial clustering of predictions from our framework, can also be of great interest to support invasion surveillance and decision-making. Environmental managers are often left wondering which time of the year specific life stages of species will occur. Most of the information available (when available) is found in technical and scientific literature and typically indicates the months or seasons of occurrence at broad geographical resolutions, for example, a country, group of countries or a continent. For example, a highly comprehensive recent work on invasive species in the forests of Europe (Veenvliet et al. 2019) highlights the months of expected occurrence of phenological stages with the highest visibility for each species across Europe as a whole. This information is much welcomed by managers and is often the best available for most species, but it well illustrates the difficulties of obtaining high temporal and spatial resolution data on the timing of phenological stages. The maps obtained with our approach go a step further, identifying these regions at the level of individual grid cells, with temporal resolutions as fine as daily and by accounting for inter-annual variability.

In conclusion, our work demonstrates the potential of widely available, temporally discrete biodiversity observation data for estimating the timing of life stages relevant to invasive species detectability. With the increasing volume of media-supported biodiversity observation data being published, the number and diversity of invasive species for which these estimates can be produced are substantial. Furthermore, these estimates can be delivered at high spatial resolutions across wide areas, in real time and for several days into the future, providing timely decision support for numerous managers tasked with planning surveillance and early detection measures. Increasing the number of invasive species covered, while continuously refining these estimates, will likely contribute significantly to global efforts in the proactive prevention of biological invasions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

CC: Conceptualisation, Methodology, Validation, Formal analysis, Resources, Data Curation, Writing - Original draft, Project administration, Funding Acquisition. AC and AM: Writing - Review and Editing.

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Data availability

Spatial weather data used for this work are publicly available online from NSF NCAR Research Data Archive (RDA) n (https://rda.ucar.edu/datasets/d084001/). Event observation data are available from USGS Non-indigenous Aquatic Species (https://nas.er.usgs.gov/queries/fact-sheet.aspx?SpeciesID=1068) and GBIF with DOIs: https://doi.org/10.15468/dl.3fve6q, https://doi.org/10.15468/dl.9drr85, https://doi.org/10.15468/dl.h5amhh, https://doi.org/10.15468/dl.9drr85, https://doi.org/10.15468/dl.h5amhh, https://doi.org/10.15468/dl.2kbnxv, https://doi.org/10.15468/dl.ycjsyz, https://doi.org/10.15468/dl.7q6rke, https://doi.org/10.15468/dl.b9sze9 and https://doi.org/10.15468/dl.uh8apz. Additional data sources and R code are publicly available on Zenodo (https://doi.org/10.5281/zenodo.13847953).

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Supplementary material 1

Additional information

Authors: César Capinha, António T. Monteiro, Ana Ceia-Hasse

Data type: docx

- Explanation note: text 1. Rationale and procedures used to address temporal recording bias. table S1. List of the 67 features used to characterize temporal environmental conditions for observation and temporal pseudo-absence records. table S2. Results from pairwise Kruskal-Wallis tests assessing significant differences in the performance of predictions from distinct algorithms modelling a distinct life stage for each of four species. fig. S1. Location of collected records of observation of imago-stage of the Geranium bronze (Cacyreus marshalli) (a); medusae of the freshwater jellyfish (Craspedacusta sowerbii) (b), flowering of the floating primrose-willow (Ludwigia peploides) (c) and flowering of garden lupine (Lupinus polyphyllus) (d), between 2016 and 2022. fig. S2. Examples of daily predictions obtained from models trained with observational data corrected for spatial bias. fig. S3. Examples of daily predictions obtained from models trained with observational data corrected for spatial and temporal bias. fig. S4. Boyce index values, corresponding to Pearson correlation values between predicted probabilities of event occurrence and the frequency of event observation records for models calibrated with data corrected for spatial and temporal bias. fig. S5. Boyce Index values for forecasts made 1, 3, 6, and 9 days in advance from models calibrated with observation data corrected for spatial and temporal bias. fig. S6. Regional patterns of predicted and observed timings of the phenological stages.
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Applications of biosensors in non-native freshwater species: a systematic review

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Abstract

Technological advances have boosted the ability to obtain large and high-quality ecological data. These new technological tools have the potential to rapidly develop knowledge on how species behave and interact in ecosystems at relevant spatial and temporal scales. Comprehensive time-series datasets on the in situ behaviour and dispersal of wild organisms are essential for addressing fundamental ecological and physiological questions regarding non-native freshwater species. In this review, we address how biosensors, hereby defined as a tool for electronic tagging and tracking, can be useful in assessing movement, internal states and behaviour in non-native freshwater species, plus information about the surrounding environment and discuss possibilities of future research.

We performed a systematic review of the available literature and retrieved a total of 132 scientific studies (from 1996 to 2023) detailing 140 examples of sensor use. Most studies used radio telemetry (40%; n = 53) followed by acoustic telemetry (34%; n = 45) and PIT telemetry (20%; n = 26) to study non-native freshwater species. The taxonomic group most studied was fish (72%; n = 109), followed by crustaceans (14%; n = 21) and amphibians (5%; n = 8). The most addressed topics included species behaviour assessment (72%; n = 101), species physiology (10%; n = 14) and management (9%; n = 12). As expected, the number of studies noticeably increased since 2006, with the majority performed in North America (55%; n = 73), followed by Europe (30%; n = 40) and Oceania (7%; n = 9). Information provided by biosensors can be used to better understand the dynamics and impacts of cryptic non-native species and can be applied in the management of biological invasions.

We also addressed future directions concerning the use of biosensors in non-native freshwater species (e.g. underwater internet systems, artificial intelligence, crittercams). Overall, these technologies provide unique possibilities in the field of biological invasions in freshwater ecosystems and the development of new technologies to address their limitations will increase the amount and reliability of the data gathered to provide information for management actions.

Key words: Biologger, biosensor, conservation, freshwater, invasive species, management, telemetry

Introduction

The introduction of non-native species has dramatically altered terrestrial and aquatic ecosystems and is now an important driver of biodiversity change, requiring enormous resources to manage their impacts (Pyšek et al. 2020; Diagne et al. 2021). Although no ecosystem is immune to the invasion of novel species, freshwater ecosystems can be particularly affected by high ecological and economic



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Copyright: [©] Alexandra Meira et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). impacts (Strayer 2010; Cuthbert et al. 2021). Ecological impacts of non-native species include the disruption of food webs, changes in nutrient cycling and modifications in physical characteristics due to ecosystem engineering, with cascading effects on biodiversity at all trophic levels, from individuals to ecosystems (Sousa et al. 2009; Gutiérrez et al. 2014; Gallardo et al. 2016). Concurrently, non-native freshwater species can generate severe economic impacts, including biofouling of structures, changes in fisheries yields and management costs, amongst others (Cuthbert et al. 2021).

The growing number of species introduced to freshwater ecosystems and their subsequent establishment highlights the urgent need for more effective measures to monitor and manage these species (Strayer 2010). However, studying non-native species in the natural environment can be challenging, particularly for species that are cryptic, shy or avoid humans or for species that colonise habitats where direct observations can be difficult (Rutz and Hays 2009). The study of non-native species in freshwater ecosystems exemplifies some of these difficulties, where high turbidity, fast river flow and deep rivers and lakes, amongst others, may make the logistics and financial investment for in-situ studies highly demanding and unrealistic. Despite possible difficulties, managers and policy-makers would benefit from a better assessment of the basic ecological and physiological features of non-native species. This information will provide insights into fundamental ecological and evolutionary questions and contribute to more efficient and effective management measures, from prevention to control, based on sound scientific data (Gurevitch et al. 2011; Ricciardi et al. 2021).

Technology has helped to overcome some of the field monitoring challenges described above by remotely measuring key ecological features of species. Technological advances in the last decade have allowed the development of new tools, enabling us to gather larger amounts and higher-quality data that can accelerate our knowledge of how individuals, populations and communities behave and interact in ecosystems (Besson et al. 2022; Costa-Pereira et al. 2022; Couzin and Heins 2022; Jetz et al. 2022). A clear example of this was the combined use of GPS tracking data and aerial imagery to assess what habitat and social features influence the movement of wild baboons (Papio anubis) in Kenya (Strandburg-Peshkin et al. 2017). In the marine realm, Goldbogen et al. (2017) applied a multi-sensor camera tag on whales to study their biomechanics. This technology allowed the determination of whale's inhalation and exhalation timing, how their feeding synchronises with depth and speed and the timing of mouth opening, maximum gape and mouth closure amongst other types of behaviour. In freshwater ecosystems, Hanssen et al. (2022) tagged Atlantic salmon (Salmo salar) smolts with a novel sensor tag to investigate mortality due to predation during the migration of smolts. These tools can also be useful to improve our understanding of the physiology, behaviour and ecology of non-native species and allow a more accurate assessment of ecological and economic impacts (Lennox et al. 2016, 2017, 2023; Katzner and Arlettaz 2020; Alós et al. 2022). In addition, some of these technologies can enhance spatial, temporal and taxonomic coverage of monitoring and have the potential to increase our understanding of the role of a determined non-native species on ecosystem functioning and dynamics. If multiple technologies can be integrated and contribute to open data, they can enable study of non-native species at different ecological levels that were previously mostly impossible.

New technological solutions are now increasingly available to better understand non-native species ecology and biosensors may be particularly useful (Lahoz-Monfort and Magrath 2021). By definition, the field of electronic tagging and tracking applies to studies where animals are remotely sensed using electronic tools (Fahlman et al. 2021). Data can either be logged or transmitted by satellite, Global System for Mobile (GSM) communications, or other means, such as radio frequency or acoustic transmissions. Dataloggers placed on organisms are referred to as biologgers, but loggers can also be placed in or above water to measure environmental variables, record sounds or images or take other remote measurements (Lahoz-Monfort and Magrath 2021). For example, Raby et al. (2020) used biosensors to compare the habitat use of the native lake trout (Salvelinus namaycush) and the non-native Chinook salmon (Oncorhynchus tshawytscha) and found both species to display different foraging tactics and behaviour, which allowed them to co-exist despite having similar diets. In the particular case of aquatic ecosystems, it should be noted that transmitters must be in the air to send data via satellite or GSM, but can send data from underwater to terrestrial fixed stations by radio or acoustic telemetry where the data are logged for later downloading; when transmitters are affixed or implanted in animals, it is often referred to as animal biotelemetry. Collectively, animal biologging and biotelemetry can be considered as a field of electronic tagging and tracking which is defined in this systematic review as biosensors.

We reviewed the bibliography available concerning the use of biosensors in non-native freshwater species to assess: i) spatial-temporal patterns of published studies; ii) the breadth of the type of sensors used; iii) and most studied taxonomic groups. We also discussed and provided preliminary insights on the potential application of these devices in advancing our understanding of freshwater biological invasions in the future.

Literature search and review

A scientific literature search was conducted using Web of Knowledge (www.webofscience.com) for published research including non-invasive sensors (i.e. sensors that do not impact species' physiology, behaviour or survival) and non-native species. While our search was conducted using Web of Knowledge (WoS) and different search bases may not cover the same publications, increasing bias, WoS provides various advantages (Falagas et al. 2008; Gusenbauer and Haddaway 2020). The search was based on an exhaustive compilation of search terms (See Suppl. material 1), including common terms (e.g. "biosensing", "invasive species", "non-native*") and specific terms for non-invasive sensors (e.g. "telemetry", "acoustic", "PIT*tag*"). We did not specify species names or genus to reduce bias. We searched only titles, keywords and abstracts and considered peer-reviewed studies discussing the use or using non-invasive sensors published up to 31 December 2023. However, only records reporting non-invasive sensors directly attached to the species' body, studies involving non-native species and studies developed on freshwater (or adjacent riparian) ecosystems were retained, while records from reviews or meta-analyses that did not add any new relevant information were excluded to avoid double counts. The publications were reviewed and discussed by the authors to ensure they were relevant to our objectives. In addition, other studies corresponding to our search, known by the authors, but that did not appear in the search results, were added to the list.

Each study was classified by sensor type used (e.g. acoustic telemetry, heart sensor, infrared sensor) and function (i.e. what it had been used for) (Table 1). In addition, data on the geographic location (i.e. country and continent), the taxonomic group of the non-native species studied (i.e. amphibian, crustacean, fish, mammal, molluscs, plant, reptile) and year of publication were also collected.

The number of papers published per year on the subject was plotted between 1996 (the year of the first record) and 2023 and a Sankey diagram was generated to illustrate the linkage amongst records on different sensor types, output and functions, based on the taxonomic group, using the R-package "networkD3" (Allaire et al. 2017). In addition, a heatmap of the total number of scientific publications on the subject per continent was also produced, using QGIS (QGIS 2023) and included charts with the percentage of research on each taxonomic group.

Function
Behaviour
Interaction
Management
Methodology
Monitoring
Physiology

Table 1. List of sensor types and function categories used in the retained studies.

General description of the dataset

After excluding records that did not match our criteria (i.e. studies not focusing on freshwater or riparian non-native species using non-invasive attached sensors), the final dataset comprised 132 scientific publications. These corresponded to 140 case studies since six publications used more than one sensor. The studies were mostly conducted in rivers (50%; n = 66), lakes (~ 27%; n = 35) and controlled freshwater environment (e.g. laboratory and mesocosms) (~ 8%; n = 11). A total of 14 different sensor types were identified and comprised acceleration data loggers, acoustic telemetry, hall sensors, heart sensors, Heat Pulse Velocity (HPV) systems, inductive proximity sensors, infrared sensors, PIT telemetry, pop-off tags, pressure sensitive tags, radio telemetry, thermal dissipation sensor, time-depth recorder and ultrasonic telemetry. Telemetry-based sensors that transmit data rather than log it on-board were the most well represented, accounting for 125 (~ 95%) studies (Fig. 1). These sensors were used to study non-native species behaviour, management, monitoring, biological interactions, physiology and survival, but also to develop methodologies to be used in future studies (Fig. 1). The majority of the 140 case studies ($\sim 72\%$; n = 101) focused on species behaviour (i.e. movement, habitat use, feeding habits and behavioural patterns), of which 18 reported a second function for the sensor (species management, n = 6; physiology, n = 5



Figure 1. Linkage amongst the relative quantity of published records using biosensors for non-native freshwater species research by major taxonomic group, type of biosensors used and functions assessed. NA refers to the Chinook salmon (*Oncorhynchus tshawytscha*) which, despite being a native species, was used to assess the impacts of a non-native predator (see Cavallo et al. (2013)). The "Other" category includes hall sensor, heart sensor, HPV system, inductive proximity sensor, infrared sensor, micro acceleration data loggers, pop-off tags, pressure sensitive tags, thermal dissipation sensor, time-depth recorder and ultrasonic telemetry.

and monitoring, n = 4 and methodology, n = 2; and interactions with other species, n = 1). Non-native fishes were the most studied taxonomic group with 108 (~ 82%) studies, followed by crustaceans (n = 21; ~ 16%), amphibians (n = 8; 6%), mammals (n = 6; ~ 5%) and molluscs and plants, each presenting three studies (~ 2%) and reptile (n = 2; ~2%) (Suppl. material 1). One paper (i.e. Cavallo et al. (2013)) studied the effects of a non-native predator by applying the sensor to the native fish species instead of the invader. The fish group included 32 non-native species, crustaceans included five, amphibians, molluscs, mammals and reptiles included only two each and plants included three (See Suppl. material 1). The most studied non-native species was *Cyprinus carpio* (n = 21; ~ 14%), followed by *Petromyzon marinus* (n = 11; ~ 7%), *Hypoph-thalmichthys molitrix* (n = 9; ~ 6%) and *Pacifastacus leniusculus* and *Ctenopharyngodon idella* (n = 8; 5%, each).

Studies included in this review were conducted in 22 countries. Most studies (55%, n = 73) were conducted in North America, the USA being the country that contributed the most with 62 studies. Of the included studies, 40 (30%) were conducted in Europe, followed by Oceania with 9 (~ 7%) (all conducted in Australia), Asia with 5 (~ 4%), Africa (South Africa) with 3 (~ 2%) and lastly, South America with 2 (~ 2%). Aside from the USA, the countries that contributed the most with research using sensors in non-native freshwater species were Canada and United Kingdom (n = 11; 8%, each), Australia (n = 9; ~ 7%, each) and the Czech Republic (n = 8; 6%). The other countries published four (~ 3%) or less studies.

Studies performed in North America focused mostly on non-native fishes (n = 73; ~ 76%; eight of the case studies used more than one species), which follow similar trends as reported in the general results (Fig. 2). Mammals were the only taxonomic group that was not studied in North America. European studies included fewer taxonomic groups, but were more evenly spread across taxa, with fishes and crustaceans the most studied groups (n = 26, 51%; and n = 19, 37%, respectively). Mammals (n = 4; ~ 8%), amphibians and molluscs (n = 1; ~ 2%, each) were less studied. Research from other continents was limited to a few taxonomic groups (Africa: fishes and plants, n = 2 and n = 1, respectively; Asia: fishes and reptiles, n = 4 and n = 1, respectively; and Oceania: amphibians and fishes, n = 6 and n = 3, respectively) or only one group (South America: mammals, n = 2).

The number of studies generally increased over time, with the earliest published paper found in 1996 (i.e. Allen et al. (1996); Fig. 3). The first study published originated from North America (USA), while records from other continents date back from 1998 (Europe; Ireland) (i.e. Donnelly et al. (1998)), 2006 (Oceania; Australia) (i.e. Brown et al. (2006)), 2012 (Asia; Japan) (i.e. Honda et al. (2012)) and 2013 (Africa and South America; South Africa, Kadye and Booth (2013); and Chile, Medina-Vogel et al. (2013), respectively). Studies from Europe and North America have been published at a constant pace, except for the years 2001, 2006 and 2011, for Europe and the years 1999–2002 and 2004–2006, for North America. Annual publication in North America averaged 2.7 publications per year, while an average of 1.5 papers from Europe was published per year since the first publication on the subject. However, while the rate of publications for Europe has been constant over the years, North America had an increase in published studies over the years, attaining the maximum of 15 publications, in 2021, compared to two publications from Europe.

Studies from North America encompass a greater diversity of sensor types (n = 11). Acoustic, PIT and radio telemetry were the most frequently applied technologies (n = 33, ~ 42%; n = 21, ~ 27%; n = 16, ~ 21%, respectively). Research in Europe was conducted using seven different sensors, with radio telemetry the most used technique (n = 26; ~ 62%). In Oceania (Australia), studies used acoustic (n = 2; 20%) and/or radio telemetry (n = 8; 80%), while research from Africa






Figure 3. Number of publications per year on the use of biosensors to study non-native freshwater species. Milestones represent the first record of the use of each identified sensor in a non-native freshwater species.

(South Africa) used either HPV systems (n = 1; ~ 33%) or acoustic telemetry (n = 2; ~ 67%). Studies from Asia used acoustic telemetry (n = 3; 60%) or acceleration data loggers or radio telemetry (n = 1; ~ 33%, each). Both studies conducted in South America used radio telemetry.

As previously mentioned, species behaviour was the most frequent focus of study (~ 72%; n = 101), whether as a unique application of sensors (n = 83) or jointly with another application (n = 18). This category includes the assessment of natural behaviour and natural movement or changes in movement in response to the environment. It was followed by species physiology (n = 14; 10%), species management (n = 12; ~ 9%), species monitoring (n = 11; ~ 8%), which included dispersal dynamics, the development of methodologies (n = 8; ~ 6%) and species interactions (n = 6; ~ 4%).

Considering the geographical distribution of the studies, some bias could be introduced and so some cautions need to be made when interpreting overall results. In fact, each country contains different environments and may be affected by different non-native species, thus displaying different priorities (Hulme et al. 2013). Consequently, this could result in focusing on different taxonomic groups as found in this review (Fig. 2) and using different biosensors, more adapted to countries' specific needs, creating an overall bias in our general results. On the other hand, the bias towards non-native fishes could be explained by this group being the most commonly introduced outside of its natural range for recreation and provisioning services (García-Berthou 2007).

Use of biosensors for studying non-native freshwater species

Acceleration data loggers

In general, accelerometers record acceleration forces in a continuous manner at a defined frequency or a defined time-average of the acceleration, being the data either stored or transmitted (Cooke et al. 2016). This type of sensor has been used to make empirical measurements of behaviour and energy expenditure mostly in terrestrial and semi-aquatic animals and fishes (Cooke et al. 2016). Acceleration Data Loggers provide detailed behavioural data, can operate in various environments, including

underwater and allow long-term monitoring. However, they present limited battery life and require retrieval of the device for data analysis (Cooke et al. 2016).

Acceleration Data Loggers can be attached to non-native species to monitor their activity levels, swimming behaviour and movement patterns in their new environments (e.g. Whitney et al. (2021)). These data can help in understanding how these species interact with their surroundings, identify patterns in their invasiveness and assist in creating control strategies.

Acoustic telemetry

Acoustic telemetry involves attaching transmitters to animals that emit acoustic pulses. These signals are detected by receivers within the waterbody. The time and location of each detected pulse allow researchers to track the movements and behaviour of aquatic species, allowing to collect high-resolution data over long time periods (Hellström et al. 2022). This technology allows real-time tracking over large areas and provides precise location data (Hellström et al. 2022). It also presents some disadvantages, such as the need for a network of receivers, which can be expensive and complex to deploy and a limited range in shallow or obstructed waters. Additionally, transmitter size may limit use to larger species (Crossin et al. 2017).

Acoustic telemetry can be used to track the movement of non-native species within freshwater ecosystems, helping to map their distribution, dispersal patterns and habitat use (e.g. Stakėnas et al. (2009); Bopp et al. (2023)). This information can be crucial for managing and mitigating the spread of these species (Crossin et al. 2017; Hellström et al. 2022).

Hall sensors and inductive proximity sensors

Hall sensors are capable of detecting magnet-transducer paired magnetic field properties (Williams et al. 2020). When a magnetic object passes near the sensor, it produces a voltage proportional to the magnetic field strength. Inductive proximity sensors detect metal objects' presence without physical contact. They work by generating an electromagnetic field and detecting disturbances caused by the presence of metal within this field (Allen et al. 1996). These sensors can be used to measure position, speed and proximity. They have high sensitivity and accuracy and are durable and reliable in a myriad of environmental conditions, while displaying low power consumption. However, hall sensors require precise calibration and the detection range is limited (Ramsden 2011).

By attaching a magnet to an animal, hall sensors can quantify its amplitude, angular velocity and frequency of limb movements, providing insights into energysaving mechanisms (Williams et al. 2020). Hall sensors and inductive proximity sensors can also measure respiration rates and extent of inhalation, heart rates, growth and even patterns of defecation, providing information on animal physiology and behaviour (e.g. Allen et al. (1996); Lorenz and Pusch (2013)).

Heart sensors

Heart sensors measure the heart rate of animals, typically using electrocardiography (ECG). These sensors are either implanted or attached to the animal to monitor cardiac activity in real-time (Cooke et al. 2004). Heart sensors can provide insights into the physiological responses of non-native species to different environmental conditions, such as temperature changes, pollution levels or interactions with native species (e.g. Kuklina et al. (2018)). These data can help assess the stress levels and overall health of non-native populations.

Heat Pulse Velocity (HPV) Systems

HPV systems measure the speed at which heat pulses travel through plant stems, which correlates with sap flow and, by extension, water transport and transpiration rates. A heat pulse is introduced and sensors measure the time it takes for the heat to travel through the stem (Burgess et al. 2001). HPV systems provide precise data on plant water use, is non-destructive and can be used over time; however, it is limited to plant studies.

HPV systems could be used to study the water usage and transpiration rates of non-native freshwater plants and plants present in the riparian area, providing data on their impact on water resources in freshwater ecosystems (e.g. Mkunyana et al. (2019)). Understanding the water consumption of these species can help in managing water resources and controlling non-native plants.

Infrared sensors

Infrared sensors detect infrared radiation (heat) emitted by objects. These sensors can measure temperature or detect movement, based on changes in the infrared radiation patterns, but also measure heart rates (Styrishave et al. 2007). Thus, they are capable of detecting species in low-light conditions without requiring physical tagging, but are limited to detecting species with significant temperature contrast to the environment.

Infrared sensors could be used to monitor the presence, activity and physiology of non-native species, particularly in nocturnal or low-visibility conditions. They can also be applied to assess physiological responses (e.g. Styrishave et al. (2007); Berry and Breithaupt (2008)).

Passive Integrated Transponder telemetry

PIT (Passive Integrated Transponder) telemetry involves the use of small, implantable tags that emit a unique code when activated by a reader's electromagnetic field. These tags do not require a battery and are often used for tracking and identifying animals. Thus, PIT telemetry can provide individual identification of tagged animals, but requires close proximity to the reader for detection, being also limited to species that can be tagged.

PIT telemetry can be used to monitor the movement, growth and survival of non-native species in freshwater environments. By tagging individuals, researchers can gather long-term data on the population dynamics, habitat requirements and spread of non-native species (e.g. Lechelt et al. (2017); Dauphinais et al. (2018)).

Pop-off tags

Pop-off tags are data-logging devices that attach to an animal and are designed to detach at a predetermined time or under specific conditions. Once released, the tag floats to the surface, where it transmits its stored data via satellite (Block et al.

1998). These tags enable long-term tracking over large distances, the data can be recovered even if the animal is not recaptured and can provide detailed information about movement and collect large amounts of environmental data (Raby et al. 2020). However, they are relatively large, limiting use to larger species, often of single-use and require specific environmental conditions for data recovery (e.g. surfacing for satellite transmission).

Pop-off tags could be used to track the movements of non-native species over long distances or periods. Once the tag detaches, researchers can recover valuable data on the species' behaviour and habitat use, which is useful for understanding their spread and impact (e.g. Raby et al. (2020)).

Pressure sensitive tags

Pressure-sensitive tags measure the pressure exerted by the surrounding environment. These tags can provide data on depth and diving behaviour by recording pressure changes over time. They can also record environmental conditions, such as temperature and can be used on a wide range of species; however, these have to dive or change depth frequently (Filmalter et al. 2015).

Pressure-sensitive tags can be used to study the diving behaviour of non-native species in freshwater ecosystems (e.g. Bajer et al. (2011)). This information can help determine the habitat preferences and potential impacts of these species on native flora and fauna at different depths.

Radio telemetry

Radio telemetry involves attaching a transmitter to an animal which then emits radio signals. These signals are detected by a receiver, allowing researchers to track the animal's location and movement in real-time. This type of telemetry is effective for studying various environments and species, but have a limited range, requiring manual tracking and researchers to be relatively close to the tagged animal (Cagnacci et al. 2010; Gussen et al. 2016).

Radio telemetry can be used can be used to monitor the movement and distribution of non-native species in freshwater ecosystems (e.g. Sammons et al. (2003); Jones and Stuart (2009)). It is particularly useful for studying species in areas where GPS or acoustic telemetry is less effective, such as areas with dense vegetation or shallow waters.

Thermal dissipation sensor

Thermal dissipation sensors measure heat loss from a surface, often used in plant studies to determine transpiration rates. The sensor measures the temperature difference between a heated probe and its surroundings, which correlates with water movement and transpiration (Granier 1985). Similar to HPV systems it is non-destructive and can be used for long-term monitoring while being limited to plant studies.

Thermal dissipation sensors can be used to study the water use and transpiration of non-native freshwater plants (e.g. Moore and Owens (2012)). These data can help assess the impact of non-native species on water resources and ecosystem health in freshwater environments.

Time-depth recorder

Time-depth recorders (TDRs) log data on the depth and duration of an animal's dives over time. These devices are attached to the animal and record depth changes, allowing researchers to analyse diving behaviour and habitat use (e.g. Hays et al. (2007)). They are suitable for long-term studies and can be used on various non-native species, but like Pressure Sensitive Tags, they are limited to species that engage in diving behaviour.

Applications and their potential for management

Biosensors used to assess movement have the potential to provide information for population dynamics and support predictions on species dispersal at relevant spatial and temporal scales. Movement of non-native species can change with time, environmental conditions and position (i.e. individuals from the core and front of the invasion), such as in the well-known case of the invasive cane toad (Bufo marinus) (Phillips et al. 2006). The dispersal of this non-native species in Australia was compared between invasion core and front using radio telemetry and evidence was found that the current dispersal rate at the front is much higher than current established populations (Alford et al. 2009). This evidence may indicate a behavioural and/or environmental change at the intraspecific (and even intra-population) level, which can lead to a better understanding of the invasion processes and the range of impacts non-native species can have on native species. Frequently, the focus is placed on impacts and biological features at the species level, including in invasion ecology. However, given the importance of environmental filtering and/or biotic resistance, these impacts or features can be highly context-dependent and should be assessed at the population level (Simberloff et al. 2013; Haubrock et al. 2024; Sousa et al. 2024). It is also important to note the type of system invaded and the connectivity between systems as the dispersal of non-native species is dependent on their ability to move to a new area. Such types of studies can only be performed in connected aquatic systems or with semi-aquatic species that can reach disconnected areas. Considering the above-mentioned case of the cane toads in Australia, we may even think of these impacts and biological features changing at the intra-population and individual levels and the use of biosensors can be extremely informative in these assessments. With the application of acoustic tags on the native Chinook salmon, Oncorhynchus tshawytscha, it was possible to evaluate the effects of non-native piscivorous fish on this species' survival (Cavallo et al. 2013). By removing the non-native predator, the survival of tagged salmon increased significantly, further highlighting the use of sensors to study the impacts of invasive species.

Non-native species adapt to the new conditions of the invaded habitats, including establishing new biological interactions with co-occurring native species. Sensors can be used to assess these new interactions and, for example, Stakenas et al. (2013) used radio telemetry to assess the possible interactions of the invasive pumpkinseed (*Lepomis gibbosus*) and the native brown trout (*Salmo trutta*). Both species occupied the same habitat; however, they presented slightly different environmental preferences with the brown trout opting for microhabitats with higher water velocity, coarser substrate and sometimes deeper waters compared with pumpkinseed. Although their range overlapped and changed similarly throughout

the seasons, the range area of brown trout was larger, with the absence of mutual attraction or avoidance, which indicates minimal to no impact of the invasive pumpkinseed on the native brown trout. Therefore, at the interspecific level, it is possible to use biosensors to assess avoidance behaviour (i.e. moving away from or avoiding the same areas as other species or conspecifics). For example, the invasive spinycheek crayfish (Faxonius limosus) and native juvenile burbot (Lota lota) have an overlapping nocturnal foraging phase. By applying PIT-tags on both crayfish and fish, researchers found that L. lota showed a strong avoidance response to the presence of crayfish. However, this behaviour was age-dependent since it was only observed in age-0 burbot, which indicates that the invasive crayfish mostly predate the young-of-the-year cohorts (Hirsch and Fischer 2008). In another case study, Salo et al. (2008) followed the movement of the non-native semi-aquatic American mink (Mustela vison) by radio-tracking and found that predation risk by a native top predator, White-tailed sea eagle (Haliaeetus albicilla), reduced mink's swimming distances. These results suggest that this behavioural change may impair mink's feeding behaviour and ultimately reduce mink population growth, mitigating its negative impacts on the invaded ecosystems.

Predation of native species can also be assessed using telemetry-based tags. Boulêtreau et al. (2020) used acoustic and radio tags on native sea lampreys (*Petro-myzon marinus*) to assess the risk of predation by the non-native European catfish (*Silurus glanis*). Tags were equipped with a biopolymer that, in case of predation, would change the tag ID making it possible to identify tagged lampreys that were eaten. The results showed that 80% of the tagged sea lampreys were preyed upon within a month and 50% were preyed on within 8 days after tagging, indicating the impacts the European catfish can have on this native species with high ecological and economic value in the studied region.

The use of sensors has also been applied to assess intra- and interspecific variation regarding environmental conditions. For example, in the United States of America, the movement of the non-native Silver carp (Hypophthalmichthys molitrix) was assessed regarding phenological and environmental factors (Coulter et al. 2016). This study found that silver carp moved upstream in the spring, before the beginning of spawning and downstream in autumn. The characteristics of the movement (i.e. movement rates, distance and direction) also changed between seasons. Despite high individual variability, the results showed patterns related to seasons and locations that could potentially be used for early detection and control. More recently, Bopp et al. (2023) conducted a similar study that used acoustic telemetry to find differences between the timing and duration of migration of the non-native Grass carp (Ctenopharyngodon idella) and the native walleye (Sander vitreus). These assessments can assist the selection of optimal timing and locations to deploy barriers and effectively block the movement of Grass carp to new habitats without affecting the native walleye. Thus, biosensors can assist the management of non-native species by providing the information needed to apply specific and effective measures. Using non-invasive infrared sensors, Styrishave et al. (2007) found that, while both native spinycheek crayfish (Astacus astacus) and non-native Signal crayfish (Pacifastacus leniusculus) displayed a similar nocturnal behaviour, P. leniusculus presented higher activity (heart rate and locomotor activity) during daytime than A. astacus. Therefore, it could be possible to use these daytime differences to target P. leniusculus for control measures.

Given the high ecological and economic impacts mediated by non-native species in freshwater ecosystems with no signs of deceleration in the number of introductions for the near future (Seebens et al. 2017), management becomes a key aspect when dealing with these species. Therefore, the application of biosensors to management could be an interesting tool that could help in the design of the best actions to prevent, detect, eradicate or control invasive species in freshwater ecosystems (Fig. 4).

Regarding prevention, the use of biosensors could give insights into the physiological tolerances of the species. For example, heart sensors have been used to record crayfish survival and recovery rates when exposed to freezing temperatures, providing insight into crayfish adaptability to different environmental conditions (Kuklina et al. 2022). These data could help generate more accurate species distribution models (SDMs) (i.e. mechanistic vs. correlative SDMs). These more accurate mechanistic models can be used to better predict areas more susceptible to invasion (Elith et al. 2010; Gallien et al. 2010; Evans et al. 2015), making this information helpful to implement prevention and early detection measures at finer spatial scales. In this scope, sensors can allow the identification of invasion fronts and this can support early detection of first invaders in particular areas. These data could be potentially useful to identify sites where traps and barriers might be deployed to effectively eradicate or at least control non-native species.

Managing the expansion of non-native species and assessing their impacts can be very laborious, expensive and inefficient. The Judas technique can be a reliable and efficient way to control and contain these invasions with cost-effective benefits compared to other ways such as fishing permits. Bajer et al. (2011) showed high removal rates (52–94%) of the common carp (*Cyprinus carpio*) from lakes in



Figure 4. The level of development of biosensors identified throughout the studies included and their potential application in the management of non-native freshwater species. The level of development was classified, based on how much is known about the sensor, its user accessibility and how recurrent its application is in the study and management of non-native species.

south-central Minnesota USA by using Judas fish that were radio-tagged coupled with pressure-sensitive tags, to locate winter aggregations of this non-native species and remove them with seine nets. Kennedy et al. (2018) showed that this technique can also be used in small flying invertebrates, such as Asian hornets (*Vespa velutina*), which are common in riparian habitats (Monceau et al. 2012). The early detection and removal of the nests is considered the only option to control the spread of this non-native species. They have used radio telemetry to successfully detect the location of the nests, by attaching a 0.28 g tag to hornet workers with a 0.8 tag: hornet weight ratio. They reported a 100% and 63% success rate in tracking the tagged hornets and previously unknown nests detection, respectively. In this case, this method can also be used for early nest detection.

Future directions

Many improvements and new approaches could be developed to exploit the full potential of biosensors to study non-native freshwater species. In this section, we share our thoughts about future directions concerning this topic following a hierarchical order, from research studies and management actions that can be implemented without new technological advances to more challenging and ambitious directions that still need further technological developments.

The number of studies published on this topic decreased after 2021. Although it has not been apparent from the last two years, possibly due to the constraints caused by the Covid-19 pandemic, it is possible there will be a significant increase in the number of studies published in the future. Despite the limited number of studies using biosensors in non-native freshwater species research, the results of these studies suggest that the use of these tools could be beneficial to investigate how non-native species interact with other global stressors, such as climate change, habitat loss and fragmentation and pollution. For example: how warming may affect the behaviour and impacts mediated by non-native species, especially those species that are poikilothermic; how the presence of physical obstacles in rivers may affect the dispersal of non-native species; and how pollution or land use may influence the physiology and behaviour of non-native species. Similar studies were conducted with native species (e.g. Moser and Lindley (2007); Hayden et al. (2014); Wilson et al. (2018)) and could, therefore, be easily adapted to non-native species. These assessments could contrast native and non-native species and determine if they respond differently to these human disturbances, which could provide information on the possible evolutionary advantages of non-native compared to native species in more disturbed ecosystems. Such approaches will likely require experimental manipulations to isolate the effects of climate (or other stressors) on the responses measured by electronic tagging, being possible outcomes relevant to better understanding the invasion process. However, mensurative approaches may also yield important outcomes if study designs are appropriately replicated, such as amongst replicated pond environments.

There is a clear geographical bias in terms of number of publications using biosensors to investigate non-native freshwater species. As referred before, some regions are under-represented, this lack of research applying biosensors to investigate non-native freshwater species being possibly caused by a lack of investment (Pyšek et al. 2008; Hulme et al. 2013). Considering the importance of non-native species at the global scale and the usefulness of biosensors in this domain, the number of studies in these regions could be increased through international collaborations, funding initiatives in under-represented regions, such as through international collaborations, funding initiatives and capacity-building programmes.

Many biosensors have some combination of the following caveats that restrict their applications, such as size/weight influencing smaller species behaviour, long-distance communication, signal interference, battery life, data storage and processing. Although some recent examples already include invertebrates, such as bivalves, in practice, this technology is mainly applied to larger animals. The current bias towards larger species found in the present review could be mitigated through the development of miniaturised sensors. Recent advancements will make this application possible for very small organisms without impairing their normal behaviour. A recent study with terrestrial gastropods showed how millimetre-sized smart sensors can be used in native and non-native snails (Bick et al. 2021) and this technology can be applied to small aquatic species. Moore and Brewer (2021) found that fishes tagged with micro-transponders (p-Chip) had higher survival than those tagged with traditional PIT tags, showing yet another advantage for the miniaturisation of sensors. Other non-invasive technologies could be applied to investigate smaller non-native species, such as environmental DNA (Barnes and Turner 2016; Brown et al. 2016); however, these methods are limited in terms of application for behavioural studies.

While some well-known and described examples of the use of biosensors in the control of invasive species exist, this type of application is only possible for the management of gregarious species or populations with low individual behavioural variability. For example, when using radio telemetry to assess the spatial behaviour of the invasive Red swamp crayfish (*Procambarus clarkii*), Gherardi et al. (2002) could not find clear movement patterns at the population level. Indeed, movement was not related to sex, hour of the day or other abiotic and biological features measured except water depth and crayfish size, which both appear to increase crayfish speed, making it impossible to develop a methodology as efficient as the Judas technique to control and possibly eradicate these populations. Nonetheless, it is essential to determine such behavioural patterns, especially as a last resort when addressing species that are already established and are, thus, unlikely to be eradicated, demanding new solutions that can only be found through relevant information collected by biosensors.

In terms of communication signalling, the information gathered is dependent on the presence of deployed structures and is highly impaired by detrimental environmental conditions. For example, radio frequency transmissions underwater can only work effectively at short distances due to their being highly affected by propagation loss, which is conditioned by salinity and temperature, amongst other environmental characteristics (Gussen et al. 2016). This shortcoming can be overcome using optical or acoustic transmissions; however, these are also dependent on environmental conditions, such as water turbidity and density. Acoustic transmissions can reach higher distances, but can have lower throughput and latency at long distances, which is dependent on water depth, salinity and temperature (Gussen et al. 2016). Thus, new systems and solutions need to be developed to solve or mitigate these limitations. One possible solution could be the application of underwater internet systems. Shihada et al. (2020) produced a system that uses off-the-shelf components that reduce the cost of production, it relies mainly on optical communications for medium distances, including LED and laser light signals and does not require additional infrastructures as it can operate using batteries and low-power devices making its use more flexible and practical. However,

limitations, such as high water turbulence, reduce the quality of the communication channel, causing interference and dispersion of the signal. Several methods similar to this have been proposed in the last few years and, while many challenges have been identified, these advancements might offer viable opportunities for underwater communication (e.g. incorporation of smart sensors to overcome and optimise the interpretation of signal noise) and data acquisition for scientific research (Kao et al. 2017; Mohsan et al. 2023).

Apart from the communication of data, there is also the problem of analysis given the amount of data gathered and stored by biosensors. Artificial intelligence (AI) could be used to support the analysis process by modelling or automatically providing information on non-native species movements and interactions using real-time transmissions with live-buoys or satellites. AI is dependent on machine-learning (ML) and has already been applied in ecological studies, such as behavioural studies (e.g. Browning et al. (2018); Norouzzadeh et al. (2018); Nath et al. (2019)), population monitoring (e.g. Norouzzadeh et al. (2018); Guirado et al. (2019)) and identification of species (Knight et al. 2017; Salamon et al. 2017; Tabak et al. 2019). The latter is often associated with community science projects through citizens assisting with the validation of the results or using smartphone applications built with AI to identify the species (McClure et al. 2020). Thus, the incorporation of AI tools in ultra-low power wireless integrated circuits with sensor automation systems or imaging of cameras (see below), augments direct applications for automotive data processing and enhances data accessibility in public databases, which could, therefore, allow faster detection responses to non-native species at a broader scale.

The use of animal-mounted cameras (crittercams) for identifying and monitoring non-native species represents a promising path for research and conservation efforts. With the integration of AI and ML, the efficiency and accuracy of species identification from crittercam footage could be greatly enhanced and the development of software capable of real-time identification could facilitate the data analysis process and provide suitable and fast feedback for conservation actions. By combining movement sensors, crittercams and ML to develop a method to automatically detect and geolocate behaviour for the flatback turtle (Natator depressus), Hounslow et al. (2023) showed its potential for management and mitigation of threats by prioritising the protection of important specific behaviour locations (i.e. rest and forage). The same strategy could be applied to protect native from non-native species. Expanding the scope of crittercams to encompass multiple species within a system could allow us to better understand the population dynamics of species and community-level impacts, which could ultimately help select meaningful management actions. Additionally, the long-term grouped application of crittercams, biosensors and AI and ML, could provide valuable insights into the ecological and evolutionary responses of native to non-native species, allowing us to understand if and how these species adapt over time and how environmental conditions affect such interactions. Some limitations need to be overcome to fully take advantage of the potential of crittercams, biosensors, AI and ML. Advances in battery life would enable prolonged studies (e.g. new ways of optimising systems' power consumption, while maintaining efficient wireless communications, self-charging systems for perpetual operations or even self-powered sensor systems that harvest energy from the surrounding environment). Establishing remote networks across distinct habitats or large areas is needed to have a comprehensive view of non-native species distribution, movements, behaviour and impacts, which demands investment and

the development of new, better and adapted solutions. In the case of crittercams, miniaturisation would be essential as currently the available technology can only apply to larger animals. Lastly and probably, the greatest issue with this technology is the elevated price, which could impair its deployment at a large scale.

By providing real-time and specific biological data, biosensors can contribute to validating and refining models and predictions related to the species studied. Ground-truthing simulations involve validating SDMs or habitat suitability models, the information gathered by biosensors on the physiology, behaviour and location being used to confirm or refute the model's predictions (Lundy et al. 2012; Blecha and Alldredge 2015). On the other hand, the data retrieved can be used to refine environmental models and improve the accuracy of simulations related to species behaviour and movement. Once again, regardless of the sensor used, these are extremely dependent on battery life and the availability of communicating stations. Previous studies already addressed the issue of battery life in real-time data transmission and suggested the use of algorithms based on accelerometer data to save battery when animals are inactive, while capturing data with higher resolution when they are active (Brown et al. 2012; Kays and Wikelski 2023).

Biosensors have been applied to detect critical environmental situations, such as pollution peaks, acting as early warning systems. For instance, by monitoring shell movement (i.e. closing time, changes in shell movement pattern and changes in valve gape), it is possible to identify the type of stressor or contaminant that bivalves are exposed to and trigger a warning signal (Kramer et al. 1989; Barile et al. 2016; Ferreira-Rodríguez et al. 2023). Following the same rationale, it could be possible to have sentinel species for non-native species. For example, by understanding the changes in behaviour in native species caused by non-native species, real-time monitoring could allow the early detection of non-native species and accelerate eradication actions. However, this will demand experimentation to decipher the signals that are specific to the responses to non-native species, which could be difficult as it might be impossible to distinguish it from the responses to other disturbances, such as pollution or the presence of a native predator, amongst other possibilities. If such a distinction is possible, then we may couple the use of sensors with AI and ML to detect these patterns.

Conclusion

The technological developments in biosensors in the last two decades provide unprecedented possibilities in the field of biological invasions in freshwater ecosystems. There is a gap in the application of biosensors to study non-native species between different taxonomic groups and bias towards telemetry-based sensors. This is probably caused by the difficulty to capture and adapt biosensors to organisms other than fishes. The potential data collected is also highly dependent on the sensor used, being unfit to serve studies on all non-native taxonomic groups. As telemetry-based sensors are the most developed and used, it is thus necessary to invest in innovation and the development of other sensors more adapted to other taxonomic groups and goals.

Considering the described limitations that biosensors still have, efforts should be prioritised towards the miniaturisation of the devices and the enhancement of battery life and real-time communication systems. The solutions developed should consider the type of study (i.e. species, environment, data, goals, invasion phase) taking into account that they might be appropriate in certain cases, but not in others. However, given the development of new technologies, including AI, underwater internet and the miniaturisation of many devices, future opportunities to monitor and manage non-native freshwater species are numerous. Nonetheless, several caveats and biases are still to be overcome, which include the study of how environmental factors (e.g. turbidity, depth, salinity) and species characteristics (e.g. size) can impair the efficacy of biosensors.

Lastly, the creation of interdisciplinary working groups involving ecologists, engineers, data scientists and policy-makers could promote the development of biosensors more suitable and effective in their applications for ecological research, enabling efficient management of non-native freshwater species.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Search strategy for ISI-Web of Knowledge database and the list of the invasive species studied with the use of biosensors and respective references

Authors: Alexandra Meira

Data type: docx

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Research Article

Benchmarking three DNA metabarcoding technologies for efficient detection of non-native cerambycid beetles in trapping collections

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Abstract

Individual sorting and identification of thousands of insects collected in mass trapping biosurveillance programmes is a labour-intensive and time-consuming process. Metabarcoding allows the simultaneous identification of multiple individuals in a single mixed sample and has the potential to expedite this process. However, detecting all the species present in a bulk sample can be challenging, especially when under-represented non-native specimens are intercepted.

In this study, we quantified the effectiveness of DNA metabarcoding at detecting exotic species within six different mock communities of native and non-native species of European xylophagous cerambycid beetles. The main objective is to compare three different sequencing technologies (MinION, Illumina and IonTorrent) to evaluate which one is the most suitable in this context. Additionally, dry and wet (monopropylene glycol and water) collection methods were compared. Although not observing significant differences in the total number of species detected amongst the three sequencing technologies, the MinION detected a greater number of species in field-like samples. All three sequencing technologies achieved success in detecting and identifying closely-related species and species in low abundance. The capture method of insects in the field greatly influenced sample preservation and detection. Individuals captured in traps containing monopropylene and water had lower DNA concentration, leading to lower species detection rates compared to individuals killed using just an insecticide without any collection medium.

Key words: Alien, biological invasions, biosecurity, Cerambycidae, exotic, Illumina[®], IonTorrent[®], Oxford Nanopore[®], xylophagous

Introduction

The exponential increase in biological invasions that has been observed over the past decades is expected to persist (Seebens et al. 2021). This is primarily due to factors such as globalisation, tourism and global warming (Chown et al. 2015). Amongst the species introduced beyond their native range by human activities, insects are the most prevalent group (Seebens et al. 2018) and can cause a wide range of impacts. Non-native insects can affect native flora, fauna and ecosystems in various ways

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(Kenis et al. 2009) and they can also transmit pathogens and diseases, thus threatening public health (Mazza et al. 2014). Economic implications are also to be considered since numerous invasive insects are important pests for agricultural crops and plantation forests, inducing huge management costs (Bradshaw et al. 2016).

Amongst these non-native insects, species associated with woody plants are increasingly dominating, accounting for 76.5% of all herbivore species newly recorded in Europe from 2000 to 2014, potentially because of the growing trade of ornamental plants and wooden packaging material transported in international cargo shipments (Aukema et al. 2010; Roques et al. 2016). One of these important families of xylophagous beetles is the long-horned Cerambycidae, with more than 200 species affecting forestry, horticulture and agriculture (Rossa and Goczał 2021), resulting in multimillion-dollar losses every year (Wang 2017). To detect potential new invasions of Cerambycids, biomonitoring programmes have been set up over large geographical areas with intensive trapping campaigns extending over several years (Roques et al. 2023; Mas et al. 2023). However, rapidly evolving trades lead to changes in trade routes and imported goods, which results in an increasing arrival of new non-native species. A high number of these species have not been previously reported as invaders; some are not considered to be pests in their native ranges and some could even be unknown to science (Seebens et al. 2018). As part of the European project HOMED (https:// homed-project.eu/), 244 Cerambycid traps were set up across Europe (France, Italy, Spain, Switzerland, Portugal, Austria, England, Greece, Slovenia, Netherlands, Bulgaria, Czech Republic and Sweden), 38 in Asia (China, Siberia, Russia), 11 in North America (USA, Canada), five in the Caribbean (Martinique) and four in Australia, all baited with generic lures for simultaneous detection of multiple species (Roques et al. 2023). In such large-scale trapping campaigns, thousands of captured insects must be sorted and identified by expert taxonomists. This identification step is time-consuming and labour-intensive, thus limiting the rapid detection of non-native individuals amongst large numbers of native ones (Piper et al. 2019; Abeynayake et al. 2021; Chua et al. 2023). Yet, it is essential that those non-native species are detected as quickly as possible to allow their eradication before establishment and dispersal (Richardson et al. 2000; Blackburn et al. 2011; Giovani et al. 2020).

For insects, traditional DNA barcoding, using a short fragment of the Cytochrome Oxidase 1 (*COI*) gene, has truly become a universal tool to identify unknown specimens at species level regardless of sex or life stage (Hebert et al. 2003). Namely, DNA barcoding has been successfully used to accurately identify cerambycid pest species for biomonitoring (Hodgetts et al. 2016; Wu et al. 2017; Kelnarova et al. 2019; Javal et al. 2021). Despite its numerous advantages, individual DNA barcoding remains a laborious and time-consuming approach in the context of mass-trapped insects, as it requires individual sorting of thousands of specimens, tissue sampling (often legs), DNA extraction and amplification and finally sequencing of each sample individually. However, the recent application of high-throughput sequencing (HTS) technologies to DNA barcoding allows for the expedited production of thousands of DNA barcodes (deWaard et al. 2019; Srivathsan et al. 2021).

This metabarcoding approach generates a large number of short DNA sequences (reads), allowing the accurate identification of multiple species simultaneously from a single mixed sample (hereafter called "bulk") (Liu et al. 2020), such as all the individual insects captured in a single biomonitoring trap. Moreover, compared to traditional morphological identification, metabarcoding offers a significant reduction in costs (Batovska et al. 2021), generally providing equivalent or better detection and identifying a much wider spectrum of taxa (Elbrecht et al. 2017; Andújar et al. 2018). Using DNA as a proxy for species detection and considering sequence variation within and amongst taxa, metabarcoding approaches are, however, constrained by the completeness of the reference databases to accurately assign sequences to correctly identified taxa (Liu et al. 2020).

Although metabarcoding has several advantages, ensuring the accuracy of detections is crucial. Erroneous detections of pest species can have severe environmental and economic consequences (Batovska et al. 2021). Yet, metabarcoding approaches still suffer from methodological limitations that may make them unsuitable for rapid biosecurity detection (contaminations, unreliable quantification, incomplete databases, false positives etc.). One specific challenge is the time required to process samples, which can suffer long delays between capturing individuals and obtaining sequencing results. This is especially true when sampling sites are located far away from laboratories or when transporting samples requires specific permits for certain species. Additionally, when external providers are slow to sequence samples, the process is further delayed. These limitations can hinder biomonitoring projects and slow down the detection of potential invasive species. As a result, the implementation of measures to mitigate their impacts may also be delayed (Krehenwinkel et al. 2019; Egeter et al. 2022). Despite these limitations, the Illumina MiSeq sequencing technology has been favoured due to its lower error rate and well-established bioinformatic procedures (Piper et al. 2019). Yet, Braukmann et al. (2019) demonstrated similar performance in sequence quality and insect species recovery using IonTorrent platforms (Ion Torrent PGM and Ion Torrent S5). As these machines are more affordable than the MiSeq, they can easily be purchased by individual laboratories. Hence, this technology is overall less often dependent on external providers and their sequencing delays.

Over the past few years, Oxford Nanopore Technologies[®] has released a very inexpensive portable sequencing platform, the MinION. This small sequencer can be connected via USB to a laptop to perform sequencing (Krehenwinkel et al. 2019) in the field and obtain sequencing data in real time conditions. Indeed, the MinION for a metabarcoding application offers the possibility of performing DNA sequencing of bulk samples directly on site without the need for transport or reliance on external sequencing providers. So far, although the MinION does not seem suitable for the characterisation of complex communities, it is already suitable for the analysis of metabarcoding data when the species diversity per sample is low and the target species are well represented in public databases (Ho et al. 2020). In addition, recent developments in Nanopore technology and base calling have reduced sequence error from 6% (Srivathsan et al. 2021) down to less than 1% (Srivathsan et al. 2024).

The primary objective of our study was to determine the most effective metabarcoding approach for the biosurveillance of Cerambycid wood-boring beetles. To achieve this, we compared the performance of three Next Generation Sequencing technologies: the portable Nanopore sequencer MinION, the Illumina MiSeq and the Ion GeneStudio S5 (IonTorrent[®]). Our evaluation focused on their ability to detect invasive species in different mock communities. Specifically, we assessed their accuracy in differentiating between closely-related cerambycid species and detecting low-abundance species in mixed-trap samples. Additionally, we analysed various metabarcoding primer pairs to evaluate their accuracy in species identification. Finally, we emphasised the significance of the field sampling protocol, particularly the trapping methods (dry versus monopropylene glycol) in species detection.

Materials and methods

Taxa sampling

Mock communities were constructed using 48 field-trapped specimens from different countries in Europe (France, Greece, Portugal, Spain), China (Beijing and Zhejiang Province) and the USA (Michigan) (Table 1), as part of a worldwide trapping experiment using multi-funnel traps baited with a generic attractant blend, including eight Cerambycid pheromones (see details of the blend composition and trapping methods in Roques et al. (2023)). Most of the specimens (36/48) were caught using a-cypermethrin insecticide (Storanet[®], BASF Pflanzenschutz Deutschland, Germany) in the trap basins, of which the bottom had been replaced with a wire mesh to allow drainage and keep specimens dry (hereafter called the "dry" method). Other specimens (12/48) were captured using a 50:50 ratio of monopropylene glycol (MPG) and water (hereafter called the "wet" method). Cerambycides collected from field-traps were stored in ethanol 95% and kept at -20 °C until further processing. Two individuals were captured by hand ("hand collected" in Table 1) and pinned in collection boxes after capture. The date, country of collection, type of trap and the 48 specimens used in mock communities are detailed in Table 1.

Mock community construction and DNA extraction

Six mock communities with varying species composition were assembled as follows:

Test 1: Identifying closely-related species.

To assess the efficiency of the different sequencing technologies and primers to differentiate between sister species, bulks 1 and 2 were composed of congeneric species (Table 1). Two legs from each individual (one specimen per species) were collected and pooled to constitute the bulks. The whole set of legs was then ground using flame-sterilised metal pestles to limit the risk of contamination. DNA from the ground material was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. Two additional legs were taken from the same specimens to assess the quantity and quality (A260/280 and A260/230 ratios) of DNA for each specimen individually (Fig. 1a).

Test 2: Detecting low abundance species.

Bulks 3 and 4 were composed of six species represented by heterogeneous DNA concentrations (Suppl. material 1: table S1) to assess the ability of the sequencing technologies and primers to detect species present in very low abundance. DNA of each individual (one specimen per species) was previously extracted using two legs that were ground as above and processed using the Qiagen DNeasy Blood and Tissue Kit. To construct bulks 3 and 4, individual DNA extracts were quantified using a fluorometer (Nanodrop[™], Thermo Fisher Scientific) and mixed together according to their concentration to achieve the needed proportions of DNA for each individual (six individuals of different species ranging from 41% to 3% for Bulk 3 and six individuals of different species ranging from 50% to 0.5% for Bulk 4) (Table 1, Fig. 1b).

Bulk	Species	Country of collection	Collection Year	Collection type
1	Arhopalus ferus	Portugal	2020	Cypermethrin insecticide (dry method)
1	Arhopalus rusticus	France	2021	Cypermethrin insecticide (dry method)
1	Arhopalus syriacus	Portugal	2019	Monopropylene glycol (wet method)
1	Xylotrechus arvicola	Portugal	2021	Cypermethrin insecticide (dry method)
1	Xylotrechus chinensis	Greece	2019	Cypermethrin insecticide (dry method)
1	Xylotrechus stebbingi	Greece	2019	Cypermethrin insecticide (dry method)
1	Xylotrechus undulatus	USA	2019	Monopropylene glycol (wet method)
2	Monochamus galloprovincialis	Portugal	2019	Monopropylene glycol (wet method)
2	Monochamus sutor	France	2019	Cypermethrin insecticide (dry method)
2	Monochamus carolinensis	USA	2019	Monopropylene glycol (wet method)
2	Monochamus scutellatus	USA	2019	Monopropylene glycol (wet method)
2	Phymatodes amoenus	USA	2019	Monopropylene glycol (wet method)
2	Phymatodes testaceus	USA	2019	Monopropylene glycol (wet method)
2	Phymatodes varius	USA	2019	Monopropylene glycol (wet method)
2	Phymatodes aereus	USA	2019	Monopropylene glycol (wet method)
2	Phymatodes dimidiatus	USA	2019	Monopropylene glycol (wet method)
3	Pyrrhidium sanguineum	France	2020	Cypermethrin insecticide (dry method)
3	Xylotrechus stebbingi	Spain	2021	Cypermethrin insecticide (dry method)
3	Monochamus galloprovincialis	Spain	2021	Cypermethrin insecticide (dry method)
3	Xylotrechus chinensis	Greece	2019	Cypermethrin insecticide (dry method)
3	Chlorophorus glabromaculatus	France	2020	Cypermethrin insecticide (dry method)
3	Phymatodes testaceus	France	2020	Cypermethrin insecticide (dry method)
4	Arhopalus ferus	France	2020	Cypermethrin insecticide (dry method)
4	Monochamus sutor	France	2019	Cypermethrin insecticide (dry method)
4	Aegomorphus francottei	France	2020	Cypermethrin insecticide (dry method)
4	Monochamus galloprovincialis	France	2018	Cypermethrin insecticide (dry method)
4	Xylotrechus stebbingi	Spain	2021	Cypermethrin insecticide (dry method)
4	Xylotrechus chinensis	Greece	2019	Cypermethrin insecticide (dry method)
5	Pyrrhidium sanguineum	France	2021	Cypermethrin insecticide (dry method)
5	Batocera rubus	China	2012	Hand collected
5	Cerambyx scopolii	France	2020	Cypermethrin insecticide (dry method)
5	Cordylomera spinicornis	France	2020	Cypermethrin insecticide (dry method)
5	Leiopus femoratus	France	2021	Cypermethrin insecticide (dry method)
5	Leiopus nebulosus	France	2020	Cypermethrin insecticide (dry method)
5	Pachyta bicuneata	China	1987	Hand collected
5	Stictoleptura cordigera	France	2021	Cypermethrin insecticide (dry method)
6	Arhopalus rusticus	France	2020	Cypermethrin insecticide (dry method)
6	Xylotrechus chinensis	Greece	2019	Cypermethrin insecticide (dry method)
6	Plagionotus detritus	France	2020	Cypermethrin insecticide (dry method)
6	Plagionotus arcuatus	France	2020	Cypermethrin insecticide (dry method)
6	Xylotrechus stebbingi	France	2020	Cypermethrin insecticide (dry method)
6	Arhopalus syriacus	France	2020	Cypermethrin insecticide (dry method)
6	Arhopalus ferus	France	2020	Cypermethrin insecticide (dry method)
6	Xylotrechus colonus	USA	2019	Monopropylene glycol (wet method)
6	Chlorophorus ruficornis	France	2021	Cypermethrin insecticide (dry method)
6	Phymatodes testaceus	France	2021	Cypermethrin insecticide (dry method)
6	Prionus coriarius	France	2010	Cypermethrin insecticide (dry method)
6	Phymatodes amoenus	USA	2019	Monopropylene glycol (wet method)

Table 1. Species, origin, date and condition of capture of the specimens used in the six bulks. Species names in bold correspond to exoticspecies. We consider specimens that have been captured on a different continent from their place of origin as exotic.

Loïs Veillat et al.: DNA metabarcoding for the detection of non-native cerambycid beetles



Figure 1. Overview of the DNA extraction protocol for tests 1 (identifying closely-related species) and 3 (mimicking field trap content on species composition) (a) and for test 2 (detecting low abundance species) (b).

Test 3: Mimicking field trap content on species composition.

Bulks 5 and 6 were built to reconstitute real trap contents by a collaborator involved in Cerambycidae trapping campaigns using multi-pheromonal traps (Roques et al. 2023). These bulks include individuals from a number of species native to Europe usually found in the traps deployed there, with the addition of non-native species that have either already been introduced or are at risk of being introduced in Europe (Bulk 5: 22 individuals of eight species, including one non-native (*Cordylomera spinicornis*); Bulk 6: 41 individuals of 12 species including two non-native ones (*Xylotrechus stebbingi* and *Xylotrechus chinensis*) (Table 1). The DNA was extracted following the same protocol as for bulks 1 and 2 where two legs were taken from each individual and ground together for DNA extraction (Fig. 1a).

PCR amplification

All bulk samples were amplified with two pairs of primers internal to the commonly used barcode fragment: BF3/BR2 (called hereafter "B") (CCHGAYATRGC-HTTYCCHCG / TCDGGRTGNCCRAARAAYCA (Elbrecht and Leese 2017; Elbrecht et al. 2019), which generates a 458 bp amplicon that was used for all the technologies; and fwhF2/fwhR2n (called hereafter "F") (GGDACWGGWT-GAACWGTWTAYCCHCC / GTRATWGCHCCDGCTARWACWGG), which generates a shorter 254 bp amplicon (Vamos et al. 2017) and was used for Illumina and MinION technologies only. Each PCR comprised 15.3 µl H₂O, 2.5 µl 10X PCR buffer, 2.5 µl dNTPs [1 mM], 1 µl of each primer [0.4 mM], 0.2 µl Dream Tag (Thermo Fisher Scientific), 0.5 µl Betaine [100 mM] and 2 µl DNA for a total of 25 µl per reaction. For both primer pairs, PCR was performed using the same programme: 95 °C for 5 min, 29 cycles of 95 °C for 30 s, 48 °C for 30 s and 72 °C for 50 s, followed by 72 °C for 5 min (Elbrecht et al. 2019). PCR products were then run on a 2% agarose gel stained with ethidium bromide and

visualised by UV transilluminator. The PCR products were then purified with the NucleoFast 96 PCR plate purification kit (Macherey-Nagel). Three PCR replicates were performed for the six bulks when using the MinION technology.

Illumina® library preparation

A second ligation PCR was performed on the products of the first PCR to add Illumina[®] tags and adapters, prepared by ligating Nextera XT indices through an eight cycle PCR (with a modified PCR protocol). The second PCR was carried out with the same conditions as for the initial PCR. Reactions (25 μ l) contained the following: 5 μ l of template DNA (purified products from the first PCR), 1 μ l of each primer [10 μ M], 5 μ l of 5X GoTaq (Promega) reaction buffer, 1 μ l of MgCl₂ [25 mM], 1 μ l of BSA [1 mg/ml], 0.5 μ l of dNTPs [5 mM], 0.125 μ l of GoTaq G2 Polymerase (Promega) and 10.375 μ l of molecular-grade water to reach 25 μ l. The PCR conditions were the same as for the first PCR, with eight cycles. The products of the second PCR were verified on a 2% agarose gel. PCR products were then equimolarly pooled into two different pools (one pool per primer pair used) and purified using the GeneJET Gel Extraction kit from an agarose gel, following the manufacturer's instructions. This library was sequenced in Illumina MiSeq using V3 chemistry (300 × 300 bp, 600 cycles) in the Sequencing Center within the Biozentrum of the Ludwig-Maximilian University in Munich (Germany).

MinION library preparation

Libraries were prepared according to the Oxford Nanopore Technologies [®] protocol: "PCR barcoding (96) amplicons (SQK-LSK110) (version: PBAC96_9114_ v110_revF_10Nov2020)" with the following specifications. After the first PCR described above, the Nanopore PCR barcoding expansion Pack 1-96 (EXP-PBC096) was used to perform the second PCR to incorporate the Oxford Nanopore Technologies [®] barcode sequences on the amplicons generated in the first PCR.

Reactions (50 μ l) contained the following: 2 μ l of template DNA (purified products from the first PCR), 0.5 μ l of each primer [10 μ M], 10 μ l of 5X GoTaq (Promega) reaction buffer, 2 μ l of MgCl₂ [25 mM], 2 μ l of BSA [1 mg/ml], 2 μ l of Q solution, 1 μ l of dNTPs [5 mM], 0.3 μ l of GoTaq G2 Polymerase (Promega) and 29.7 μ l of molecular-grade water to reach 25 μ l. The thermocycling conditions followed the manufacturer recommendations: 95 °C for 3 min, followed by 15 cycles of 95 °C for 15 s, 62 °C for 15 s and 65 °C for 30 s and 65 °C for 7 min.

Final PCR products were then quantified using Qubit and equimolarly pooled before being purified with Agencourt AMPure XP beads (Beckam Coutler). The final pool was then sequenced on the MinION sequencer (Mk1c; Oxford Nanopore Technologies °, UK) using a R10.3 flowcell (MIN111) with 1331 pores available and the LSK110 ligation sequencing kit. The two replicates of bulk 6 using the MinION technology were of insufficient quality (Nanodrop) and were, therefore, removed from the analysis.

IonTorrent[®] library preparation

For the production of the libraries, we started with 5 ng of DNA extract (Qubit measurement). The Nextflex Cellfree DNAseq kit (PerkinElmer) was used for the process. The quality of the libraries was assessed using Qubit (for quantification)

and Bioanalyzer (using the HighSensitivity kit from Agilent, for size verification). After quality control, each library was amplified by emulsion PCR on the Ion One Touch 2 instrument, with a concentration of 15 pg/ μ l. Subsequently, the libraries were sequenced on an Ion GeneStudio S5 system using a single-end sequencing protocol with a 300 bp read length. Sequencing was performed on an Ion 520 Chip by the GeT-BioPuces platform (Toulouse, France).

False positive detections

False positive detections are considered to be the detection of a species within a bulk that was not initially present when the bulks were constructed. In order to estimate the representativeness of false positives within true positives in the bulks, the total number of reads assigned to false positive OTUs was reconciled and compared to the number of reads assigned to non-false positive detections. The number of false positives detected according to the different tested combinations is indicated in Suppl. material 1: table S2.

Reference barcode dataset

A dataset was built using all the public sequences of all Cerambycidae species available in BOLD systems v.4 (Ratnasingham and Hebert 2007). It was then verified whether all 33 species present in the bulk samples were represented by at least one sequence in the database. Three species not previously included in the database were barcoded through Sanger sequencing on an ABI 3500 genetic analyser (Applied Biosystems) using the Big-Dye Terminator V3.1 sequencing kit (Applied Biosystems) and BF3/BR2 primer pair. They were subsequently added to our local database to ensure that they were represented by at least one barcode sequence. The number of sequences in the database for each species is shown in Suppl. material 1: table S1.

The final reference dataset is available from BOLD in the dataset DS-MINION (dx.doi.org/10.5883/DS-MINION) and includes one barcode per species together with the three newly-generated barcodes. Lab Sheet from the DS-MinION database is shown in Suppl. material 1: table S3.

Illumina® data processing

The raw data were analysed using the FROGS v.4.0.1 pipeline, a standardised pipeline containing a set of tools that are used to process amplicon reads that have been produced from Illumina[®] sequencing (Escudié et al. 2018; Henrie et al. 2022). First, amplicons with a size between 408 and 508 for the BF3/BR2 primer pair and 204 and 304 for the fwhF2/fwhR2n primer pair were retained using the Pre-process tool. For this first step, paired-end reads are merged using VSEARCH (Rognes et al. 2016) with a mismatch rate set at 10%. Cutadapt (Martin 2011) is then used to remove sequences in which both primers are absent and to trim the primers. Sequence clustering was then performed using the SWARM algorithm (Mahé et al. 2014) with a maximum sequence difference set at d = 1 (--distance 1 parameter), as recommended by SWARM. Chimeric sequences were then removed with the Remove chimera tool relying on VSEARCH with the de novo UCHIME method (Edgar et al. 2011; Rognes et al. 2016). Sequences were aligned to the same

database used for the MinION and IonTorrent[®] data analysis. In order to remove all spurious detections, OTU detections with fewer than 10 reads were removed. In barcoding and metabarcoding studies of insects, the sequence similarity level for OTU identification usually ranges from 95% to 99% (e.g. Gibson et al. (2014); Zenker et al. (2016)). We calculated the best threshold value for our dataset by applying the function localMinima from the R package spider v.1.5.0 (Brown et al. 2012). Based on this analysis, we used a threshold of 98% to assign OTUs to species level with blastn+ (Camacho et al. 2009). The resulting OTU tables for Illumina F and Illumina B are provided in Suppl. material 1: tables S4, S5, respectively.

MinION and IonTorrent® data processing

Bioinformatics analyses were performed on the Genotoul Bioinformatics Platform (INRAE, Toulouse, France). Basecalling and demultiplexing were performed for MinION data using Guppy v.6.1.7; ONT; high accuracy base calling mode; parameters: -c dna_r10.3_450bps_hac.cfg --min_qscore 5 --trim_barcodes. Then, for MinION and IonTorrent[®] data, we used the msi data processing pipeline v.0.3.6 (Egeter et al. 2022) to reduce the error rate of the reads by polishing them after the basecalling step. Reads smaller than 40 bp were removed with cutadapt v.4.0 (Martin 2011). The size range was set between 408 bp and 508 bp for BF3/BR2 and between 204 bp and 304 bp for fwhF2/fwhR2n. The clustering step was carried out with ISONCLUST v.0.0.6.1 (Sahlin and Medvedev 2020; with parameters: --mapped_ threshold 0.825 and --aligned_threshold 0.55) and a consensus sequence per cluster was generated using RACON v.1.5.0 (Vaser et al. 2017). The polished reads were then clustered at 97% sequence identity with CD-HIT v.4.8.1 (Fu et al. 2012) and a representative sequence from each cluster (centroid) was selected. The polished reads were then aligned to the local database with BLAST (BLASTn algorithm). The following parameters were used: -word_size 11 -perc_identity 95 -qcov_hsp_perc 98 -gapopen 0 -gapextend 2 -reward 1 -penalty 1 -max_target_seqs 100. Similarly, to the Illumina® data processing, OTU detections with less than 10 reads were removed. Finally, a taxonomic assignment was performed for each query using a Lowest Common Ancestor (LCA) approach with the bioinformatics package metabinkit (Chain et al. 2016; Egeter et al. 2018; Kitson et al. 2019) with the following thresholds: 98% at species level, 97% at genus level, 95% at family level (Alberdi et al. 2018; Egeter et al. 2022). The resulting OTU tables for MinION B, MinION F and IonTorrent B are provided in Suppl. material 1: tables S6-S8, respectively.

Statistical analysis

A two-sample test of proportions was used to compare and assess the significance of the proportion of reads assigned to the species levels for MinION, Illumina and IonTorrent technologies using the "Social Science Statistics" website (https://www. socscistatistics.com/tests/anova/default2.aspx). The proportion of reads assigned to different taxonomic levels was calculated by summing the total reads from different bulk samples for each condition. To determine if the number of false positives was significantly different amongst the three technologies and the two primer pairs, we calculated the detection mean for each bulk under different conditions. We then performed an ANOVA test followed by a Tukey HSD test using the "Social Science Statistics" website. The Wilcoxon test, Exact Fisher's test and standard deviation were calculated in R v.4.3.2 (https://www.R-project.org/). Sensibility, which measures the ability to correctly identify true positives, was calculated using the following formula: true positives / (true positives + false negatives) and precision, which measures the ability to measure the proportion of correct detections, was calculated using the following formula: true positives / (true positive + false positives).

Results

A total of 1,248,595 reads were sequenced with the MinION Nanopore[®] technology using the F primer pair, with an average of 78,037 (SD = 28,415) reads per sample. After quality filtering and removal of reads of incorrect size or insufficient quality, 1,113,844 (89.2%) reads were retained, with an average of 69,615 (SD = 25,508) reads per bulk. For the B primer pair, a total of 1,132,604 reads were sequenced, with an average of 62,922 (SD = 17,442) reads per sample. After quality filtering, a total of 948,832 (83.8%) reads were retained, with an average of 52,712 (SD = 14,512) reads per bulk (Table 2).

Table 2. Number of raw reads obtained after sequencing and after pre-processing steps according to sequencing technologies and primer pairs used.

Technology	Primer pair	n_raw_reads	n_reads_post_filtering
MinION	В	1,132,604	948,832
_	F	1,248,595	1,113,844
Illumina	В	1,549,894	1,025,637
-	F	2,383,028	1,686,058
IonTorrent	В	838,489	280,695

The Illumina[®] sequencing produced a total of 1,549,894 reads using the B primer pair, with an average of 258,316 (SD = 39,365) reads per bulk. After quality filtering, 1,025,637 (66.2%) reads were retained, with an average of 170,940 (SD = 69,961) reads per bulk. For the F primer pair, a total of 2,383,028 reads were sequenced, with an average of 397,171 (SD = 84,482) reads per bulk. After quality filtering, 1,686,058 (73.3%) reads were retained, with an average of 281,010 (SD = 112,512) reads per bulk (Table 2).

Regarding the IonTorrent^{\circ} technology, 838,489 reads were sequenced, with an average of 139,748 (SD = 17,086) reads per bulk with the B primer pair. After quality filtering, 280,695 (33.5%) reads remain, with an average of 46,782 (SD = 5,025) reads per bulk (Table 2).

Benchmarking of sequencing technologies

The MinION technology accurately identified 28 out of 48 specimens at the species level, Illumina[®] technology allowed specific identification of 27 specimens and IonTorrent[®] identified 24 specimens. The primer pair F enabled the specific identification of 27 specimens at species level, while the primer pair B enabled the identification of 31 specimens at species level. Illumina[®] F, Illumina[®] B and MinION B allowed for 25 species-level identifications across all bulks (sensibility = 0.52) and 24 for MinION F and for IonTorrent[®] B (sensibility = 0.50). This difference was not significant (Fisher's Exact Test, p = 1.00) (Fig. 2).

The number of reads assigned at the species level was significantly higher with Illumina[®] technology (p.value < 0.00001) compared to MinION. Nearly 97% of reads were assigned at the species level for the Illumina[®] F combination compared to 90% for the MinION F combination (p.value < 0.0001). As for primer pair BF3/BR2, over 87.3% of reads were assigned at the species level for Illumina[®], followed by over 79.7% for MinION technology and over 77.2% for IonTorrent[®] technology (Fig. 3). The primer pair fwhF2/fwhR2n resulted in a significantly higher percentage of reads assigned at the species level (93.6%) (considering both Illumina[®] and MinION technologies) compared to the couple of primers B (81.4%) (considering all three technologies) (p. value < 0.00001). The summary of the number of reads is shown in Suppl. material 1: table S9a, while the percentage is shown in Suppl. material 1: tables display data assigned to the taxonomic levels of species, genus, family or higher, for all conditions tested.



Figure 2. Upset plot showing the number of individuals detected at species level according to the three technologies (Illumina, MinION and IonTorrent), primer pairs (F=fwhF2/fwhR2n [254 bp]) and B=BF3/BR2 [458 bp]) and technology-primer pair combinations tested.





False positive detections (i.e. a species detected within a bulk that is not part of the bulk's initial composition) were observed regardless of the combination of primers and technology (Fig. 4). The precision (the ability to measures the proportion of correct detections) of the Illumina F and MinION F combinations are 0.625 and 0.667, respectively, while for the Illumina B, MinION B and IonTorrent B combinations, the precision is 0.806, 0.926 and 0.8 respectively. An average of 13.5 false positive OTUs were recorded for the primer pair fwhF2/fwhR2n, compared to an average of four false positive OTUs when using the primer pair BF3/BR2, the difference being significant here (p value = 0.00194). According to the technology used, but regardless of the primers, an average of ten, seven and six false positives were recorded for Illumina[®], Min-ION and IonTorrent[®] technologies, respectively. There are no significant differences amongst the three sequencing technologies in terms of false positives.

Mock community analysis

In total, 33 out of 48 individuals (68.8%) were detected at the species level by at least one experimental condition (Fig. 5).

Bulks 1 and 2 were assembled to compare the detection rates of closely-related species under different sequencing and primer conditions. Illumina[®] detected seven species out of 16 (43.75%), MinION also detected seven out of 16 (43.75%) and IonTorrent[®] detected six species out of 16 (37.5%). No significant differences were observed amongst the different methods used (Krustal-Wallis chi-squared = 2, df = 2, p value = 0.3679).

Metabarcoding of bulks 3 and 4 aimed at comparing the ability of different sequencing technologies to detect low abundance species in the traps. All sequencing technology/primer combinations allowed for the detection of minor species: *Phymatodes testaceus* with a presence of 3% in bulk 3 (relative amount of DNA in the mock community) and *Xylotrechus chinensis* with a percentage of 0.5% in bulk 4. However, some species (although not in minority in the bulks) were not detected in one or several tests (Fig. 5). In total, Illumina[®] was able to detect a higher number of individuals (11/12 individuals detected) compared to MinION (9/12) and IonTorrent[®] (9/12).

Regarding bulks mimicking the species composition in a field trap, MinION performed better to detect and identify specimens at the species level in Bulk 6



Total number of false positive detections at species-level for each sequencing platforms and primers used

Figure 4. Number of false positive detections at species level for each sequencing platform and primers used (F=fwhF2/fwhR2n [254 bp] and B=BF3/BR2 [458 bp]).



Figure 5. Heatmap comparing the identification of individuals present in bulk samples at the species level (green square) or the absence of detection at the species level (grey square) according to the sequencing technologies and primer pairs used (F=fwhF2/FwhR2n; B = BF3/ BR2). Species names written in blue were collected using the wet method, those in green were collected using the dry method and those in dark red were hand-captured.

(detecting8/12species(66.7%)) compared to Illumina[®] and IonTorrent[®] technologies (5/12 species (41.7%)), whereas the same number of species was detected for Bulk 5 (4/6 (66.7%)) regardless of the technology used. Nevertheless, in bulk 5, the non-native species, *Cordylomera spinicornis* was detected only by Illumina B. For bulk 6, the non-native species *Xylotrechus chinensis* was detected by all three technologies and *Xylotrechus stebbingi* by MinION B only.

Impact of capture and storage conditions on individual detection

Our results demonstrate significant differences in the mean number of detections between samples that were collected using the "dry" method (α -cypermethrin insecticide) and the "wet" method (water-diluted propylene glycol) (Wilcoxon rank-sum test, W = 74.5, p value = 0.0006342) (Fig. 6A). Indeed, 75% (9/12) of specimens collected using the "wet" MPG trapping procedure were not detected by any of the sequencing technologies. Conversely, 88.2% (30/34) of those collected using the "dry" trapping procedure (based on α -cypermethrin insecticide) were detected at least once across all technologies.

Individuals captured using the "dry" method had higher DNA concentration (39 ng/µl on average (SD = 52.79)) than MPG trapped specimens (18.6 ng/µl on average (SD = 21.80)) (Wilcoxon rank-sum test, W = 123.5, p value = 0.04533) (Fig. 6B). The average A260/280 ratio was 1.9 for the "dry" method and 2 for the MPG method (Fig. 6C). However, the average A260/230 ratio of specimens trapped with the "dry" method (0.8) was higher than that of specimens captured with MPG (0.5) (Wilcoxon rank-sum test, W = 146, p value = 0.1502) (Fig. 6D).

Discussion

Rapid and precise detection of exotic insects is crucial to prevent the ecological and economic damage they can cause by invading new environments and disrupting local ecosystems.



Figure 6. Boxplots representing (**A**) the average number of detections according to the type of preservation used, (**B**) the natural logarithm scale (base e) of the average DNA concentration according to the type of preservation used, (**C**) the A260/280 quality ratio according to the type of preservation used and (**D**) the A260/230 quality ratio according to the type of preservation used. The black dots represent the outlier values (values outside the whiskers). The bold line represents the average value, outlines of the boxes represent the first and third quartiles and the whiskers represent the range of the values outside the quartiles.

Benchmarking of sequencing technologies

A slightly higher number of individuals were detected and identified to species using MinION (28 specimens) compared to Illumina® (27 specimens) or IonTorrent® (24 specimens), although this difference is not significant. However, this result demonstrates that the sequencing error rates long attributed to the MinION did not impact detection rates, while allowing for the elimination of the long delays often required when sequencing is performed on other sequencing technologies (Piper et al. 2019). It must be considered that we worked on a single pair of primers (BF3/BR2) with the IonTorrent[®] technology, which may have reduced the number of identifications. More specifically, our results showed that the choice of primer pairs and the length of the amplicon generated led to contrasted results regarding taxonomic assignment. For example, only BF3/BR2 allowed the species-level identification of the invasive species Xylotrechus stebbingi. This difference may be due to the longer amplicon generated by this primer pair, which has more informative nucleotide sites to provide a reliable taxonomic assignment. By contrast, fwhF2/ fwhR2n generated a significantly higher number of false positives than BF3/BR2 (Fig. 4). This may be because the amplicon generated by fwhF2/fwhR2n is smaller in size compared to BF3/BR2. As a result, any loss of genetic information is more likely to result in misidentification or false positives (Meusnier et al. 2008).

Regardless of the number of identified species, the Illumina[®] technology produced a higher percentage of reads allowing species-level identification compared to MinION or IonTorrent[®]. The detection of specimens at a higher taxonomic level (genus or family) can be explained by sequencing errors that produce reads with less than 98% identity to the reference database. These results confirm that Illumina has a lower sequencing error rate than Oxford Nanopore's MinION sequencer (Piper et al. 2019), although this did not impact the number of individuals identified to the species level. The three technologies showed similar efficiency in detecting and identifying closely-related species. Moreover, the results show that all three sequencing technologies (regardless of the associated primer pairs) enabled the detection and identification of species whose DNA represented a very low percentage in the mock community (Fig. 5). This high resolution would allow for the detection of exotic species that are poorly represented in traps, which might otherwise go unnoticed. Thus, all three technologies appear suitable for detecting and identifying species present in low numbers in field traps, enabling effective monitoring.

Impacts of capture and storage conditions on DNA conservation

Both the conditions of capture (wet versus dry methods) and storage (i.e. time lag between collection and lab processing) have an impact on DNA concentration and quality and subsequently on the rate of species detection (Piper et al. 2019). Thus, the number of species detected is highly variable between bulks 1 and 2, which can be explained by the capture methods used: 'dry,' where individuals were captured without preservative fluid (as is the case for the majority of detected individuals comprising bulk 1) and 'wet,' where individuals were preserved in 50% MPG until trap retrieval (as is the case for the majority of undetected individuals comprising bulk 2). For instance, the species Phymatodes testaceus was always detected (10 out of 10 assays) when dry specimens were present, even in low concentrations (3% in Bulk 3). On the other hand, wet specimens of P.testaceus were rarely detected (one detection out of five assays). Individuals captured using the MPG method had lower DNA concentration and presented significantly lower detection rates compared to individuals captured using the "dry" method. Ballare et al. (2019) also found that insects collected in propylene glycol traps produced lower quality ddRADseq assemblages than specimens collected by net sampling and directly transferred into 100% ethanol (EtOH) or by passive trapping followed by 100% EtOH storage before pinning. In contrast to this, Ferro and Park (2013) found that propylene glycol is an effective DNA preservative for molecular marker-based studies on Coleoptera species. However, in their study, insects were first killed and preserved in 100% ethanol before being stored in glycol, while in our study insects were killed directly in propylene glycol. The use of 100% ethanol as the initial killing agent may lead to better initial preservation of specimens compared to direct exposure to propylene glycol.

False positives, negatives and unmatched OTUs

Despite the precautions taken, several false positives were detected in all tested conditions. The number of false positives was significantly higher with the primer pair fwhF2/fwhR2n, which generates a smaller size amplicon compared to BF3/BR2. Even though Illumina technology is known to have a lower sequencing error rate compared to MinION (Silvestre-Ryan and Holmes 2021), our study found 10 false positives generated by Illumina, while MinION produced seven false positives and IonTorrent produced six. The sensitivity of HTS technologies allows for the detection of very small amounts of DNA, thus detecting even the slightest cross-contamination between samples (Liu et al. 2020). These DNA contaminations may have occurred during sample collection in the field or in the laboratory through cross-contamination between samples from the same study.

The false negative detections for some individuals may primarily be explained by the highly heterogeneous DNA quality of the different sequenced individuals (Suppl. material 1: table S1). In fact, DNA quality can be impacted by numerous mainly abiotic factors (pH, UV radiation, temperature), degrading DNA quality in a matter of days or weeks (Strickler et al. 2015; Collins et al. 2018; Harrison et al. 2019). During field trapping using stationary traps, captured insects are sometimes exposed to such conditions (high temperatures in trap containers when exposed to the sun in summer, high humidity in the container during heavy rains etc...), which can greatly accelerate the speed of DNA degradation in captured individuals. Such degraded DNA is more difficult to amplify, thus generating false negatives, especially when attempting to detect insects in low abundance within a trap, such as an invasive species in the process of establishing (Preston et al. 2022). Another possible cause for the high number of false negatives is the bias induced by PCR, such as uneven amplification of the DNA of the different individuals present in one sample (Preston et al. 2022). To avoid potential bias arising from identification errors or missing species in the reference databases, we decided to work on a local and curated BLAST database. However, when target species are partially unknown, as is the case in field conditions, analyses must rely on public reference databases. Yet, out of the 35,000 known species of Cerambycidae to date, only 2,926 species (8.4%) are recorded in BOLD with a barcode fragment (as of 16 November 2023). Furthermore, databases can contain errors such as mis-assignment of a DNA sequence to a wrong species due to morphological identification errors. This was precisely the error encountered for the species Monochamus sutor, which was genetically identified as Monochamus sartor (Suppl. material 1: table S7) or the species Leiopus nebulosus which has been genetically identified as Leiopus linnei (Suppl. material 1: table S7) using our local BOLD database.

One also needs to pay attention to synonymy whereby a species appears in the database under multiple names. We encountered this problem in our analysis with *Arhopalus ferus* (Bulks 1, 4 and 6) which was detected, but under the name of *Arhopalus tristis* (Suppl. material 1: table S10). Finally, mitochondrial paralogues such as NUMTs (non-functional copies of mitochondrial genes transported into the nuclear genome) present in databases can also bias results, making it impossible to identify specimens correctly at the species level (Bensasson et al. 2001). NUMTs are numerous in many organisms, including some cerambycids such as *Monochamus galloprovincialis* (Koutroumpa et al. 2009; Haran et al. 2015).

The differences in identification or non-detection between morphologically similar species belonging to the same genus, as observed, for example, with Mono-chamus spp., Phymatodes spp. or Arhopalus spp. (Fig. 5), can be explained in part by the reasons mentioned above.

Biases

Based on the results obtained, it appears that the main biases observed in metabarcoding analyses of trap contents stem from the degradation of DNA from individuals, which generates false negatives. We recommend favouring a "dry" rather than a "wet" trapping method, especially the MPG method and to plan for the collection, transportation and processing of captured individuals as soon as possible after capture. This includes checking the traps as frequently as possible (at least once a week), thus avoiding excessively long exposure of the individuals
to unfavourable environmental conditions. Once individuals are brought back to the laboratory and if DNA cannot be extracted straight away, it is important to limit any further degradation by keeping samples at -20 °C and in 95% ethanol. On the other hand, DNA extractions should be stored in the preservation buffer provided with the extraction kits or in molecular-grade water and kept at -20 °C (Preston et al. 2022). We also recommend limiting the use of primer pairs that generate short amplicons, which can favour the amplification of non-target taxa, NUMTs and lead to identification errors. The quality and completeness of the databases are also very important bias factors. To limit this bias, Egeter et al. (2022) recommended restricting the database used to targeted species in order to minimise the risk of false positives due to contamination. Limited taxonomic and geographical coverage of sequence databases is a huge limitation in metabarcoding studies. For example, Dopheide et al. (2019) found no representative sequence in the GenBank database for more than 900 invertebrate OTUs in their study when analysing the community of soil arthropods from a native forest in Ireland. Additionally, species identification errors and cases of synonymy lead to false negatives or cases of multiple affiliations.

Conclusion

By comparing the accuracy and detection capacity of three metabarcoding strategies, this study contributes to improving our toolkit for monitoring non-native insect invasion. All three sequencing technologies performed equally well and showed similar results for detecting and identifying exotic Cerambycid species collected in field traps. However, MinION stands out as a portable, easy-to-use, and cost-effective sequencer, with the potential to become an essential tool for biodiversity monitoring projects. Using MinION reduces the time spent on laboratory handling compared to Illumina and eliminates the need to outsource sample sequencing. This saves considerable time when it comes to detecting invasive species. The MinION technology is accurate enough to detect non-native species even when present at low abundances in field traps and allows for accurate identifications as long as there is a sufficiently complete high-quality reference database to avoid identification errors or false positives/negatives. It is also crucial to pay close attention to issues of contamination and specimen preservation during and after individual capture in order to work with the least degraded DNA possible.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Loïs Veillat, Géraldine Roux, Carlos Lopez-Vaamonde and Stéphane Boyer conceived the study. Alain Roques collected field samples. Stéphane Boyer, Marina Querejeta, Emmanuelle Magnoux and Loïs Veillat conducted the laboratory sample processing. Loïs Veillat analysed the data and wrote the first draft. All authors contributed to the preparation of the manuscript. Both senior authors, Géraldine Roux and Carlos Lopez-Vaamonde, contributed equally to this study.

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Data availability

Barcode data for the 33 species used in the mock community experiment are available from BOLD in the dataset DS-MINION (dx.doi.org/10.5883/DS-MINION). Raw sequence data for this project and analytical script and files are available on figshare (https://figshare.com/projects/DNA_metabarcod-ing_an_efficient_way_to_detect_non-native_cerambycid_beetles_in_trapping_collections_/171432).

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Supplementary material 1

Additional data (OTU tables, samples metadatas; summary tables; ...)

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Data type: xlsx

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Data Paper

Predicted occurrence and abundance habitat suitability of invasive plants in the contiguous United States: updates for the INHABIT web tool

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Abstract

Invasive plant species have substantial negative ecological and economic impacts. Geographic information on the potential and actual distributions of invasive plants is critical for their effective management. For many regions, numerous sources of predictive geographic information exist for invasive plants, often in the form of outputs from species distribution models (SDMs). The creation of a repository of consistently produced SDMs of regional- or national-scale information predicting the potential distribution of invasive plant species could provide information to managers in the prioritisation of invasive species management. Here, we present a novel set of not only habitat suitability models for occurrence for 259 manager requested invasive plant species in the contiguous United States (USA), but also habitat suitability models for abundance (≥ 5% cover) and high abundance (≥ 25% cover). These data provide an update to the Invasive Species Habitat Tool (INHABIT; gis. usgs.gov/inhabit). This tool contains information on the majority of invasive plant species in the contiguous USA with sufficient location data for model building. INHABIT provides a canonical set of predicted geographic distributions for invasive plants in the contiguous USA that can aid in the search for new populations of invasive plant species and help create watch lists for emerging invaders. As this tool contains information on nearly all of the most problematic invasive plants in the contiguous USA, it helps in prioritising management strategies by showing which plants are already present or abundant in a land management area and which may become present or abundant in the future.

Key words: Early detection and rapid response, land management, species distribution modelling, watch list

Introduction

Invasive species cause considerable damage to ecological and economic systems worldwide (Bellard et al. 2016; Diagne et al. 2021; Mayfield et al. 2021). In the United States of America (USA), this results in an annual cost exceeding \$19 billion per year (Fantle-Lepczyk et al. 2022). The effective management of invasive plants relies on information about where particular plant species are present and abundant (Wallace and Bargeron 2022) and where they are likely to become established and abundant in the future (Mainali et al. 2015). Proactive management of invasive species is often considered the most cost-effective strategy for control,



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but this strategy is likely underemployed (Cuthbert et al. 2022), in part due to the lack of predictive tools and data to guide searches for invasive species and the prioritisation of management actions.

Predictive habitat mapping, based on species distribution models (SDMs), is the primary tool used to anticipate where invasive plant species will establish and become abundant (Crall et al. 2013; Elith 2017). SDMs can be useful in guiding management if the models used meet the standards needed for particular management objectives (Sofaer et al. 2019). Often, the lack of consistent methods, species or geographic scope can make it difficult to use or compare disparate models to prioritise the management and detection of invasive plant species. While there is often a perceived trade-off between the geographic scope and precision with SDMs, evidence suggests otherwise. Even at local scales, range-wide models often outperform ecoregion extent models of predicted species distributions, likely due to larger datasets that represent a species' niche more accurately (Jarnevich et al. 2022). The practical value of publicly available, high-quality SDMs across large spatial extents, such as the contiguous USA and at a fine spatial grain (~ 100 m²) for a comprehensive set of invasive plant species, is widely recognised amongst invasive species managers (Engelstad et al. 2022).

Most SDMs predict the habitats and geographic locations in which an invasive species might establish (i.e. become present). However, managers also want to know where species might become abundant as abundance is correlated with impact (Sofaer et al. 2018; Bradley et al. 2019; Pearse et al. 2019). To address this, SDMs can be fitted with sites where an invasive plant species is known to reach some threshold of abundance (e.g. over 25% cover; Jarnevich et al. (2021); Beaury et al. (2023); Evans et al. (2024)). Some management strategies require information about different thresholds of abundance to prioritise the application of control methods (Yokomizo et al. 2009). For example, areas of the Great Basin are twice as likely to burn when exceeding a threshold of 15% cover of invasive cheatgrass (Bromus tectorum) compared to habitats where cheatgrass is less abundant (Bradley et al. 2018). Additionally, while cheatgrass occurs in all 50 States, it is only in the western USA where it reaches a level of abundance that makes it a species of concern. Management strategies that focus on cheatgrass control for fire mitigation may choose to concentrate efforts on regions exceeding this threshold for cheatgrass cover. While many management actions rely on information about the abundance of an invasive plant species, information on where the species is likely to establish, even at low abundances, is critical for tracking dispersal. This is especially true for early detection and rapid response (EDRR) strategies that focus on the control of non-native species before they become abundant. Likewise, control of non-abundant invasive plant species may be a desirable proactive measure because the geographic locations of habitats in which an invasive plant species may become abundant are likely shifting due to changing climate (Evans et al. 2024).

Effective management of invasive plant species requires the prioritisation of species to control (Kumschick et al. 2012). Globally, more than 13,000 plant species have been introduced and established outside of their native ranges (van Kleunen et al. 2015). Within the USA, many of these introduced species do not become problematic and exist within the novel range with relatively few negative consequences (Bradley et al. 2024). However, a percentage of those introduced plant species become invasive, which we define as a non-native species that cause harm to the environment, economy or human, animal or plant health. Comprehensive information about all invasive plant species in a region can aid in prioritising management decisions. For example, managers of particular land management areas are especially interested in those invasive plant species that have not yet been detected within their management area, but which have the potential to establish there (Jarnevich et al. 2023a). We developed the Invasive Species Habitat Tool (INHABIT; gis.usgs.gov/inhabit) to provide a consistent, comprehensive set of habitat suitability models for a wide variety of invasive plant species to provide this information to managers (Jarnevich et al. 2023a).

In this data paper, we present the first abundance-based suitability models for a large suite of plant species through version 4 of INHABIT and describe the methodology used in its creation. This dataset is the first publicly available resource to provide a large number of predicted habitat suitability maps and management area summary tables for the habitats in which invasive plants may establish and may become abundant. The scope of the tool is invasive terrestrial plant species in the contiguous USA and includes 23% of all introduced vascular plant species with at least 100 georeferenced records and 50% of those with high abundance records (Fig. 1). As the inclusion of plant species in INHABIT was based on requests from diverse invasive species managers over a period of five years, it includes



Figure 1. The total number of non-native vascular plant species on the US Register for Introduced and Invasive Species (RIIS; Simpson et al. 2022) with different filtering steps applied for occurrence (top) and abundance (bottom) records, with the count of species remaining at each step reported. Filtering steps include removing species flagged as aquatic based on inclusion in species tracked in the U.S. Geological Survey's Non-Indigenous Aquatic Species (NAS; Terrestrial) and followed by whether any records from the target background dataset from aggregated data sources have been collected after 1980. Occurrence record filtering includes number of species with at least 100 occurrence records post 1980. The abundance filtering includes successive criteria for abundance records for different percent cover thresholds and record counts. The last step for both is the number of remaining species modelled for INHABIT, demonstrating the much higher capture of non-native plant species in the USA with recorded abundance populations then the number of occurrence records or the total on the US RIIS list.

nearly all of the non-native plant species considered most problematic by managers within the contiguous USA. The tool uses a nested tabular design to serve management-relevant information on plant species' distributions and displays details, such as model performance, continuous suitability surfaces and environmental predictors of suitable habitat for each species. We additionally make suitability maps easily downloadable for user-selected species and provide simple methods for importing those maps into the platforms commonly used by land managers to guide searches for new populations of invasive plants (Suppl. material 1: supplement 2, Field Maps instruction). INHABIT provides the most comprehensive predicted habitat suitability information for both occurrence and abundance of invasive plants in the contiguous USA and serves as a blueprint for modelling and data delivery in other regions.

Methods

The model fitting and summarisation methodology described below represents elements first described by Young et al. (2020), iterated by Engelstad et al. (2022) and used in version 3 of INHABIT by Jarnevich et al. (2023b). For version 4 of INHABIT, we have further updated the methods used to produce version 3. Differences between versions 3 and 4 include an increased number of species, updated occurrence records through 2023, spatial cross-validation of models and models of abundance and high abundance suitability along with occurrence suitability. All code is written in R version 4.4. (R Core Team 2024) and scripts are available in Suppl. material 1: supplement 3. Model inputs and outputs are available from Jarnevich et al. (2024) at https://doi.org/10.5066/P14HNEJF.

Species data

We asked people managing invasive plant species within the contiguous USA to contribute to a list of terrestrial non-native plant species to include in version 4 of INHABIT. The resulting list identified 286 non-native species. We obtained species occurrence and abundance data for these selected species from existing aggregated occurrence and agency databases (Suppl. material 1: table S1), including: the Early Detection and Distribution Mapping System (EDDMapS), the Bureau of Land Management's (BLM) Assessment, Inventory and Management (AIM) and Lotic databases, the BLM and National Park Service's National Invasive Species Information Management System (NISIMS), a published aggregated plant dataset (Bradley et al. 2024), the Standardised Plant Community with Introduced Status (SPCIS) database (Petri et al. 2023), the LANDFIRE reference database and the Goldwater weeds database for both types of data and the Global Biodiversity Information Facility (GBIF) and iMapInvasives database for occurrence only (see Suppl. material 1: table S1 for additional details). We used the Integrated Taxonomic Information System (ITIS; www.itis.gov) as a taxonomic authority to obtain all synonyms for all species using the R package "taxizedb" (Chamberlain et al. 2023).

We restricted species records and observations based on multiple geographic and data quality criteria. We retained records with observation dates \geq 1980, listed as "observation" or "specimen only" observation types (GBIF only) and a coordinate uncertainty \leq 30 m. Additionally, we used the "CoordinateCleaner" package (Zizka et al. 2019) to flag and remove potentially erroneous records, including those located near country capitals, country centroids, GBIF headquarters and known biodiversity institutions (e.g. research centres, universities, herbaria, museums, zoos and botanical gardens), or coordinates over oceans or other potential data errors (e.g. latitude and longitudes equal to zero, latitudes and longitudes with identical values).

We also obtained locations for all vascular plant species included on the U.S. Register of Introduced and Invasive Species (US-RIIS) list ver. 2.0 (Simpson et al. 2022) using the same criteria for inclusion. These data were used to control for sampling biases as detailed in the modelling description below. The US-RIIS documents introduced (non-native) species that are established (reproducing). We filtered this list to those at the species level (removed hybrids and sub-species) and limited it to vascular plant species (phylum Tracheophyta). These data were separated into two files, one with all georeferenced observations for the selected US-RI-IS species, and another further filtered to observations that contained abundance information for species within this list.

Modelling group

The greatest advance for version 4 of the INHABIT webtool is the inclusion of SDMs that predict species abundance in addition to SDMs that predict occurrence. Through informal discussions with managers related to previous work with occurrence and abundance habitat suitability models (occurrence, abundance as > 10% cover; Jarnevich et al. (2021)) (occurrence and four abundance groups; Beaury et al. (2023)), we decided to include three groups of habitat suitability models in version 4: occurrence (or presence), abundance and high abundance. Manger feedback indicated the two categories were two few (Jarnevich et al. 2021) and the five categories too many (Beaury et al. 2023) and most people providing feedback desired a low abundance category (around 5%) and a category around 20%. We settled on data classification into occurrence, abundance (\geq 5% cover) and high abundance (\geq 25% cover) categories, based on that manager input and data availability (cover class bins commonly found in input datasets where 25% was a more commonly used cut-off than 20%; Suppl. material 1: fig. S1).

Each observation record was classified into high abundance ($\geq 25\%$ cover), abundance ($\geq 5\% - 25\%$ cover) or occurrence (< 5% cover or no abundance information). Where numerical cover data were provided, we assigned abundance categories to those records. When there were numerical bins, we used the minimum cover in the bin as a conservative match to our categories, so that 5–30% would be assigned to the abundance bin. However, some aggregated occurrence databases included qualitative descriptions of abundance, which we manually classified: occurrence = "trace", "rare", "sparse", "single plant", "spot", "light", "low"; abundance = "medium", "moderate", "common", "patch", "patchy", "scattered dense patches"; high abundance = "high", "dense", "abundant", "heavy", "major", "dense monoculture", "dominant cover".

We used a nested set of observation records to fit models. Occurrence suitability models included observation records for a species from all three categories. Abundance suitability models were fitted with occurrence records from both the abundance and high abundance categories (\geq 5% cover). High abundance suitability models only used observations categorised as high abundance (\geq 25% cover).

Data preparation

We spatially thinned species records by reducing observations to a minimum 900 m distance between points using the "geoThin" function in the R package "enmSd-mX" (Smith et al. 2023) to limit spatial autocorrelation while preferentially retaining points with the highest abundance cover class. Additionally, we removed records falling within waterbodies by using our percentage clay soils predictor as a mask because the soil layers did not have values in areas of water (Suppl. material 1: table S2).

We required at least 100 spatially thinned observations within the contiguous USA to generate an occurrence model for a species. Any requested species with fewer observations were flagged for future modelling efforts using globally sourced observations and predictor data. For abundance and high abundance models, we required at least 50 spatially thinned observations for each model group and we only considered fitting abundance models for species for which we could fit occurrence models. Through previous INHABIT iterations, we have found it difficult to fit models with less than 50 locations and, unlike occurrence data beyond the USA, we are unaware of global repositories that include abundance information.

In statistical and machine learning communities (Hastie et al. 2009; Lever et al. 2016; Kuhn and Johnson 2019), best practices for model building advocate splitting data into three tiers including train, test and sub-setting train into k-fold cross-validation splits. The train data are used to fit the model, with the cross-validation subsets providing information for model refinement. The test partition is used to evaluate model performance as this partition is fully withheld from the model fitting and refinement process. We implemented a hashtag-based method to split each species' data into non-random, spatially sampled cross-validation (CV) data for model training and a separate withheld test dataset to evaluate model performance. This methodology uses a hashtag shape (#) overlaid on a 99% binary kernel density estimate (KDE) of the observations. Observation points falling within the buffered hashtag were assigned to the test split (and withheld from model fitting to be used for model evaluation) with a desired ratio of 70% train/30% test. The width of the hashtag test strips was manually adjusted from the default of 30% of the binary KDE extent on a case-by-case basis as needed to stay within the range of 20-50% of observations in the test split (ideally around 30%). The training data were further split spatially, based on the hashtag shape into nine CV splits. We used a single hashtag shape to define the spatial splits (both test and CV) for all three model groups and, thus, the test data for abundance were a subset of occurrence and for high abundance were a subset of abundance. We did not include a test split in the few cases where that would result in < 100 observations to train the occurrence model (n = 5 species, Suppl. material 1: table S3), though these still had a CV split. In cases of high geographic clustering resulting in algorithms failing to fit models with the spatial CV splits, we instead applied a randomised 9-fold CV split (i.e. no spatial CV-split).

As we did not have absence data, we required background locations to capture the environments available to each species to fit the models. We used two methods to generate background points for occurrence model training data to fit two sets of models for each species to account for sampling biases, a continuous KDE method and a target background approach. The continuous KDE method has been suggested for invasive species in particular because there may be a higher density of observations in a region to which the species has been introduced longer compared to the density of observations in a region where a species has only arrived recently

(Elith et al. 2010), whereas the target background approach is meant to mimic bias in where people are collecting observations (Phillips et al. 2009). For the KDE method, we created a continuous KDE raster around the observations that were used to weight the generation of an initial 15,000 random background points. We spatially thinned the background points in the same manner as the occurrence records, randomly selecting 10,000 of the remaining points. For the target background method, we randomly selected up to 10,000 locations of non-native vascular plant observations (from the US-RIIS list) within the matching lifeform of four possible lifeforms assigned by the USDA Plants Database (forb/herb/vine, graminoid, shrub/vine or tree) and restricted to the same 99% binary KDE used for the hashtag splits. As there is a relationship between residence time and abundance, we chose to only use the target background approach for the abundance models, assuming that these models will suffer less from issues related to sampling density due to residence time and more from sampling bias related to where people are making observations. We selected background points from the abundance observations of the non-native plants from within the same broad lifeform category. For all three model groups, training data background points were excluded from the buffered hashtag-defined test extent and were assigned to CV splits using the same spatial designation as for the occurrence/abundance records employed for the individual species as described above. For test splits, we generated one set of background points by rasterising the buffered hashtag shape and subsampling 10,000 randomly distributed points separated by at least 900 metres from within this shape. Thus, all model groups had the same background test points for each species.

Environmental data

We used 52 of the 54 environmental predictors included in INHABIT version 3 (Jarnevich et al. 2023b), representing a range of environmental factors including topography, temperature, atmospheric water, landscape water, soil properties, disturbance (including fire and anthropogenic disturbance), biotic interactions (e.g. tree cover and bare ground) and radiation for the contiguous USA. These predictors were used as inputs for version 4 models after they were modified using the PARC (Project, Aggregate, Resample, Clip) module in the Software for Assisted Habitat Modelling (SAHM, v. 2.2.2; Morisette et al. 2013) to ensure all predictors were in the same coordinate reference system (ESRI:102008), spatial resolution (~ 100 m²), extent and alignment. Observation data for each species were combined with predictor data to create a merged dataset representing the environmental conditions at each presence and background location used as an input in SAHM. For more information on individual predictors, their units of measure and spatial and temporal resolution, see Suppl. material 1: table S2.

For each species, we selected predictors based on individual species characteristics including biology and lifeform (e.g. winter annual graminoid) and invaded geographic distribution within the contiguous USA. Predictor sets were consistent between occurrence models (i.e. between the KDE background approach and target background approach) and were kept identical between abundance and high abundance models, except in a few cases where the number of predictors for high abundance were highly restricted based on number of observations. To avoid autocorrelation amongst potential predictors, we assessed the degree of correlation using the Pearson, Spearman and Kendall's pairwise correlation tests and removing one of any pair with a correlation coefficient > 0.70 (maximum of Pearson, Spearman or Kendall; Dormann et al. (2013)). We also removed any predictor that did not make ecological sense for a particular species (e.g. ratio of March precipitation for a tree species occurring in the eastern USA). In addition, we preferentially retained predictors known to be important for the distribution of invasive plants in the contiguous USA, specifically minimum winter temperature and index of human modification (Williams et al. 2024). We maintained a ratio of at least 10:1 observations to predictors (Hosmer and Lemeshow 2000), rounding up to the nearest ten. This resulted in a range of 10 to 29 predictors (mean 23.8 predictors) for each species for model fitting for occurrence models and a range of 5–31 predictors (mean 17.9 predictors) for each species for model fitting for abundance and high abundance models.

Model fitting

Following the methodology in Young et al. (2020) and Engelstad et al. (2022), we fit five different algorithms in SAHM including boosted regression trees (Elith et al. 2008), generalised linear models (McCullagh and Nelder 1989), multivariate adaptive regression splines (Elith and Leathwick 2007), Maxent (Phillips et al. 2017) and random forests (Breiman 2001). We updated the SAHM downsampling code for random forests to balance the number of bootstrapped samples from each class (presence and background) and improved model performance over default settings (Valavi et al. 2022). We fit models with observation training data delineated by the hashtag (see Suppl. material 1: table S3 for individual sample sizes and number of cross-validation splits). We examined each algorithm's output, using a priori criteria to identify model overfitting, including instances when the difference between the Area Under the Curve (AUC) calculated for training data and the average cross-validation AUC was > 0.05 or visual assessment of response curves appearing overly complex. In these cases, we explored alternative algorithm-specific tuning parameters to decrease the AUC difference and response curve complexity (see Suppl. material 1: table S3 for individual model changes). Finally, we assessed model performance using the continuous Boyce index (CBI; Hirzel et al. (2006)) calculated for each algorithm for each model per species and the AUC, revisiting any model where train or test CBI < 0.5 or AUC < 0.7 to reconsider parameterisation and dropping models with poor performance and fit.

Spatial outputs: maps and ensembles

For each model group, we created continuous spatial predictions of relative habitat suitability across the contiguous USA at ~ 100 m² spatial resolution, expedited by U.S. Geological Survey high performance computing resources (Falgout et al. 2024). We also calculated a multivariate environmental similarities surface (MESS) to highlight areas of model extrapolation that could be applied as a mask to any output maps (Elith et al. 2010). The MESS map compares the value for each predictor at a location to the range of values for the predictor within the training set and we highlight locations with negative values that indicate at least one predictor included in a model set had a value outside those of the training data, termed novel environmental conditions. As we had background points rather than absence data, models were not necessarily calibrated to each other. Therefore, we rescaled the mapped values for each model between 0 and 100 to make the maps more comparable. We then produced ensemble maps for each model group by taking a mean of the rescaled relative habitat suitability values, weighted by individual model CBI values (using the test CBI, if available). Individual models with CBI values of less than 0.50 were assessed and dropped if deemed appropriate, based on examining models for the specific species considering how low CBI was, how it compared to other algorithm predictions and ecological plausibility of response curves and variable importance (see notes column in Suppl. material 1: table S3 for specific reasons for retaining or dropping individual algorithms).

Through informal discussions with managers during presentations, managers expressed interest in having categorical maps of suitability along with continuous relative predictions of suitability for the three model groups. They reported an interest in three options as existed on INHABIT version 3, ranging from more inclusive to more restrictive. Thus, we produced three distinct binary versions corresponding to each of the three continuous maps (occurrence, abundance, high abundance) using percentile thresholds of 1%, 5% and 10% to convey a gradient of inclusive (comprehensive) to restrictive (targeted) model output (Engelstad et al. 2022). A percentile threshold is calculated by extracting predicted values for all training observations and selecting the predicted value for the observation that would misclassify the set percentage of positive observations. Finally, we combined the binary maps to display information across all three model groups (occurrence, abundance, high abundance) for each of the three thresholds, while highlighting any areas of environmental extrapolation.

Tabular outputs: zonal summaries and distance measurements

Managers can utilise summaries of habitat suitability for management areas in various ways, such as to create watch lists (e.g. Jarnevich et al. 2023a) or find out how many management areas have suitable habitat for a particular species. INHABIT version 3 included tables summarising mapped outputs to quantify area of suitability and distance to closest records for some federal lands (Bureau of Land Management, Fish and Wildlife Service, National Park Service, U.S. Forest Service) and U.S. Counties. For INHABIT version 4, we obtained 10,216 management area polygons to meet stakeholder, manager and agency needs including additional federally owned lands (U.S. Army Corps of Engineers, Department of Defense service branches), State Parks and Forests, sub-basins (8-digit hydrologic unit code) and other more specific land management areas, such as cooperative invasive species management areas. Descriptions of the management areas and the additional processing steps necessary to prepare them for analyses are detailed in Suppl. material 1: table S4.

We summarised the categorical maps for management areas (Suppl. material 1: table S4) using the "exactextractr" R package (Baston 2023). For each species and management area, we calculated the total land area of suitable habitat for each of the three model groups (occurrence, abundance, high abundance) for each of the three thresholds (1%, 5%, 10%). Values were nested such that summaries of occurrence models included locations defined as suitable by any of the three model groups. We also calculated the total area that was identified by the MESS maps as having novel environmental conditions (areas of extrapolation as identified by any single predictor having a value outside the range of the model training data for that predictor). To provide further information on uncertainty, we calculated the area of a management area that was available for modelling, removing the parts of

the management area that we were unable to make predictions for which typically represented water, but potentially other locations where all predictors did not have data (though this is exceedingly rare).

We also generated an updated dataset containing the modelled species' observation locations to capture the most recent observations following the same steps outlined in the species data section above. Using these data, we counted the number of occurrence locations within each management area boundary and measured the distance from boundaries to the nearest location when no observations fell within the management area.

We merged the habitat suitability summary information with the count and distance information to provide information for watch list development. Early detection at a local level can be informed by watch lists of doorstep invaders, which we define as species with habitat suitability in the focal area, with no known records within the area and with records within either a 50- or 100-mile (75–150 km) buffer of the area (Jarnevich et al. 2023a). As early detection may be most effective in preventing establishment following secondary spread into a new region, we have added a new set of larger areas. We included summaries for ecoregions, watersheds and state boundaries that, when combined with other management areas, can identify species with suitable habitat within a management area which are not yet found within the larger region.

Results

Managers requested models of 286 species. Of these 286 species, 254 species had at least 143 observations for a spatial test/train data split and five had at least 100 filtered observations for a model to be fitted with no test split. Twenty-seven species had < 100 filtered occurrences and, therefore, did not have models fit. Additionally, 217 species had at least 50 abundance observations (\geq 5% cover) and 189 had at least 50 high abundance observations (\geq 25% cover).

Overall, models performed well with most CBI values, based on the withheld test data, > 0.75 for both individual algorithms and ensemble models for all three model groups (Fig. 2). There were 16 species that had at least one algorithm excluded from the ensemble due to poor occurrence model performance (typically CBI < 0.5). This number was 25 for abundance models and 18 for high abundance models. This resulted in 4933 maps produced to develop the 665 continuous ensemble prediction maps and 259 categorical maps for each of the three thresholds. Based on an evaluation rubric for species distribution models, the models were categorised as acceptable or ideal (Suppl. material 1 table S5; Sofaer et al. 2019).

The continuous maps display relative habitat suitability at a ~ 100 m² resolution for the contiguous USA. There is a relative suitability map for each model group, including occurrence, abundance and high abundance (see examples in Fig. 3). While the example in Fig. 3 includes masking of areas of environmental extrapolation, both the available data and the webtool also include map versions without masking. Habitat suitability patterns differed between species. For example, *Ulex europaeus* (common gorse) had suitability concentrated in the southeast and northwest coast of the USA (Fig. 3a–c), while *Tamarix chinensis/ramosissima* (combination of two species of tamarisk that are difficult to distinguish and hybridise) had suitability concentrated in the west (Fig. 3d–f). *Tamarix chinensis/ramosissima* also had a large, pronounced difference in suitability for occurrence versus abundance or high abundance in the eastern USA.



Figure 2. Histograms of the continuous Boyce Index (CBI) for all fit models across algorithms and species calculated for the **a** training data **b** withheld test data and for the continuous ensemble models for the **c** training data and **d** withheld test data.

The integrated maps illustrated the differences in the thresholds used to generate them and showed differences in patterns between species (Fig. 4). By definition, the 1st percentile maps (Fig. 4a–d) contained a larger area of suitable habitat for each group than the 10th percentile maps (Fig. 4c, f). For our example species, *Ulex europaeus* showed a marked decrease in occurrence suitability and even the most targeted threshold contained visually discernible amounts in all categories of suitability (Fig. 3a–c). However, *T. chinensis/ramosissima* had much less occurrence suitability across all three maps, with areas of suitability for high abundance covering the largest area, indicating that, in most areas where the species could establish, it could also become abundant (Fig. 4d–f).

From the tabular summaries, across all species and management areas with suitability, the mean percentage suitable area ranged from 20% to 46% for occurrence, 11% to 26% for abundance and 7% to 19% for high abundance. See management summary tables in Jarnevich et al. (2024). The lower numbers correspond to data from the most restrictive threshold ("first"; derived from maps such as Fig. 3a, d) and the higher numbers correspond to the most inclusive threshold ("tenth"; derived from maps such as Fig. 3c, f). Of the model summaries, for 10216 management areas, 41 lacked predictions for at least one species due to a lack of information for at least one predictor.



Figure 3. Continuous ensemble map for each of three model groups representing low to high habitat suitability for *Ulex europaeus* (**a**–**c**) and *Tamarix chinensis/ramosissima* (**d**–**f**) including **a**, **d** occurrence **b**, **e** abundance (\geq 5% cover) and **c**, **f** high abundance (\geq 25% cover). Black indicates areas with novel environmental conditions (values for at least one model predictor outside the range of values captured by the training data for model fitting).

Discussion

We created updated models of habitat suitability for occurrence for 220 species, new occurrence models for 39 species and models of abundance habitat suitability for 217 species (Suppl. material 1: table S3, Fig. 1). These models are currently fitted using binary classification of abundance and demonstrate the potential for invasive species to reach high abundance and, hence, become problematic in new areas. As higher quality continuous abundance data become available across larger spatial extents, future models may be created showing continuous predictions for invasive plant abundance (e.g. Sofaer et al. 2022). Information regarding critical cover thresholds relating to impacts is often not available for individual invasive plant species, thus, more research in this area would improve abundance threshold selection and better inform managers about the ecological threats invasive plants pose at different levels of abundance.

Model applications for management and decision-making relevant to invasive plants are diverse. Regional early detection and rapid response applications allow for newly-introduced or actively expanding invasive plant species to be monitored prior to establishment in a management area. Similarly, an "invaders at the doorstep" approach uses





models to develop watch lists that could provide information for early detection efforts for species found nearby, but not yet within a management area. Abundance models can be similarly used for management applications, but can be applied to further refine surveys or control efforts to prioritise those species that may be more impactful in an area. When used in conjunction with spatial data on vegetation and wildlife resources, managers may better identify intersections between areas that support greater invasive plant abundance and areas with particularly vulnerable native communities. Managers may choose to prioritise actions based on model outputs and landscape features, such as when an area that is predicted to support higher abundance of an invasive plant is positioned next to a road or waterway that may further spread propagules.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: All; Data curation: CSJ, PE, DW, KS, CR; Formal analysis: all; Funding acquisition: CSJ, JSP, ISP; Investigation: all, Methodology: all, Project administration: CSJ, PE; Software: PE, DW, KS, CR, GH; Resources: CSJ;, Supervision: CSJ; Validation: CSJ, PE, DW, KS; Visualization: PE, DW, KS, CR; Writing – original draft: all; Writing – review & editing: all.

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Data availability

All of the data that support the findings of this study and the study outputs are available in the main text, Supplementary Information or as a U.S. Geological Survey data release (Jarnevich et al. 2024; https://doi.org/10.5066/P92476V6).

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Supplementary material 1

Additional information

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Data type: docx

- Explanation note: **supplement 1.** Supplementary figures and tables. **supplement 2.** Field Maps instructions. **supplement 3.** Data processing R scripts.
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Research Article

Even the losers: five-year distribution dynamics of alien plant species in South African savanna

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Abstract

We studied the short-term dynamics of the occurrence of alien plant species in a South African savanna. Within the MOSAIK (Monitoring Savanna Biodiversity in the Kruger National Park) project, plant species were recorded in a representative set of 60 plots, 50 m × 50 m in size, across the entire KNP in 2019–2020, distributed to cover a range of savanna habitats, i.e. perennial rivers, seasonal rivers and dry crests. The sampling focusing on alien plants was carried out in the same plots in 2024 and the changes in distribution patterns that occurred over the 4-5 years since the first sampling were assessed. In the first sampling period, 23 alien species were recorded and, in the second sampling, 20 were recorded; this gives a total of 25 alien species over the whole period of 2019-2024. In the recent survey, Alternanthera pungens, Conyza bonariensis, Gomphrena celosioides, Bidens biternata and Achyranthes aspera were most widespread, present in at least 10 plots. Using log-linear models, we showed that the total number of alien species records in plots did not significantly differ between the two sampling periods, indicating the absence of trends in species richness for the alien flora of KNP. There was a highly significant effect of habitat, with sites at perennial rivers harbouring more alien species than those at seasonal rivers and on crests. We also found a marginally significant interaction of habitat and sampling period, reflecting that the dry crests currently harbour fewer aliens than in 2019–2020. The frequency of some of the most invasive KNP species, such as Parthenium hysterophorus, Xanthium strumarium and Opuntia stricta, remained basically the same. However, Conyza bonariensis is an alien species that was quite rare in studied plots in 2019-2024, but its presence dramatically increased and it became widespread and locally abundant beyond the surveyed plots in some parts of KNP. Although not too successful until a few years ago, this species represents a future plant invasion threat in KNP.

Key words: Africa, dry savanna, longitudinal data, non-native species, plant invasions, riverine habitats

Introduction

Plant invasions are amongst the most significant threats to global biodiversity and protected areas are no exception (Foxcroft et al. 2013, 2017; Pyšek et al. 2020b; IPBES 2023). Even though biological invasions affect biota in protected areas worldwide (e.g. Goodman 2003; Pauchard and Alaback 2004; Padmanaba et al. 2017; Pyšek et al. 2020b), a concern that dates back to the 1860s (Foxcroft et al. 2017), very few protected areas have good baseline information and only a handful



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Copyright: © Petr Pyšek et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0) of those that are well-studied have robust invasive alien species lists available (see IPBES 2023 and examples referred therein).

Although studies reported from several parts of the world that some protected areas act as a barrier against the spread of alien plant species (Pyšek et al. 2003; Foxcroft et al. 2011; Gallardo et al. 2017), most are vulnerable to invasions and only rarely are they entirely free of alien species (Foxcroft et al. 2017; Moodley et al. 2020). The presence of alien invaders brings about a variety of impacts on species, communities and protected ecosystem that include the alteration of habitats, ecosystem regime shifts and losses to native species abundance, diversity and richness (Foxcroft et al. 2013; Hulme et al. 2014; Pyšek et al. 2020b; Novoa et al. 2021). Invasive plants are also regarded as a significant threat by the managers of protected areas (Pyšek et al. 2013; Shackleton et al. 2020). Of various taxonomic groups of invasive organisms, plants posed the greatest continued threat in a study analysing trends over ~ 30 years from the 1980s to the present, where their species numbers increased in 31% of the protected areas (Shackleton et al. 2020).

Most data on the occurrence of alien and invasive plants recorded in the field are collected in a particular place only once. Yet, longitudinal field data proved most helpful in providing information on invasion dynamics. If such data, based on permanent plots and repeated sampling, exist, they are primarily used to determine the spread of the populations of invasive species (Müllerová et al. 2005) and monitor changes in their impact (Yurkonis and Meiners 2004; Jäger et al. 2009; Dostál et al. 2013). Longitudinal data are, however, also crucial for assessing the dynamics of alien species participation in plant communities over time at a fine-grain scale (Miller et al. 2021; Kermavnar and Kutnar 2024) and identifying factors driving these dynamics (Staudhammer et al. 2015).

In the Kruger National Park, South Africa, a significant threat from alien plant invasions to the savanna ecosystem is associated with rivers that act as the most efficient pathways for propagules from adjacent areas (Pyšek et al. 2020a; Foxcroft et al. 2023; Hejda et al. 2023). Rivers and associated riparian habitats have been repeatedly demonstrated to harbour disproportionally more alien species, including some remarkable invaders, because of strong propagule pressure, supply of nutrients and provision of safe sites for germination and establishment (Pyšek and Prach 1993; Planty-Tabacchi et al. 1996; Foxcroft et al. 2007; Richardson et al. 2007; Pyšek et al. 2010).

In other systems, alien invaders that were confined to riverbanks and riparian areas for a long time have started to spread to surrounding environments, such as *Impatiens glandulifera* in central Europe (Čuda et al. 2017, 2020). However, the data collection in KNP has focused so far on alien species hotspots in human-disturbed habitats, such as tourist camps and other infrastructure or riverbeds. Systematically investigating distributions of alien plants across the entire park would allow us to assess how successfully they persist in various habitats and whether spreading from riverbeds also happens in KNP. Still, such information was missing until a few years ago (Pyšek et al. 2020a). To close this gap, an effort was made within the MOSAIK (Monitoring Savanna Biodiversity in the Kruger National Park) project, which is focused on studying biodiversity across the entire KNP (Delabye et al. 2022; Hejda et al. 2022; Čuda et al. 2024). There, we assessed to what extent alien plants are confined to rivers as the primary introduction pathway and dispersal vector versus how commonly they occur in drier habitats away from rivers (Pyšek et al. 2020a).

Here, we explore the fine-scale dynamics of alien species occurrences over 4–5 years in the South African savanna, based on repeated sampling of the same plots. This research has been motivated by the fact that it is unknown: (i) how great the fluctuation in their presence is across various habitats, i.e. how much their contribution to overall plant species richness is changing over time, (ii) what is the effect of habitat (i.e. by perennial rivers, seasonal rivers and on dry crest) on these dynamics and on alien species persistence and (iii) how stable are the populations of individual species. At the time of the first sampling (2019–2020), the covers of alien species in plant communities were generally low (Pyšek et al. 2020a), which is in sharp contrast to riverbeds in KNP where the invasions are large-scale, stable and persistent (Hejda et al. 2022, 2023). Thus, it is legitimate to ask how persistent are these small populations of alien species beyond riverbeds, how much they fluctuate or shift across savanna and whether some of these apparent "losers" have the potential to generate future threats to savanna biodiversity.

Methods

Study area

Kruger National Park (KNP), established in 1898, is the largest game reserve in South Africa and one of the oldest national parks in the world (Carruthers 1995). It is located in the north-eastern part of the country, covering an area of 19,169 km² and stretching ~ 450 km north-south and 84 km east-west. The majority of KNP has a subtropical climate, with the Tropic of Capricorn crossing the Park in the north and several perennial rivers flow through the Park, mainly in a west-east direction (i.e. Sabie, Olifants, Crocodile, Letaba, Shingwedzi, Luvuvhu and Limpopo; Fig. 1). The Park has diverse geological conditions (granitoid bedrock in the western vs. volcanic, mainly basalt and gabbro, in the eastern part), altitude (140–780 m a.s.l.), climate (450–750 mm of annual precipitation) and vegetation (dominant woody species, proportional representation of woody cover vs. open grassland; MacFadyen et al. 2016).

A recent update of the alien flora of KNP focused on species that occur in natural areas in KNP (i.e. beyond tourist camps and other infrastructure) and, thus, represent a potential threat to the diversity of native species. This work identified 146 alien plant taxa, of which 30 are casuals, 58 are naturalised, 21 have become invasive (in the sense of Richardson et al. 2000, i.e. rapidly spreading across the Park) and 37 with unresolved status (see Foxcroft et al. 2023, also for details on the assessment of invasion status of particular taxa). Twelve of the invasive species in KNP are globally widespread and five (i.e. *Pontederia crassipes, Lantana camara, Opuntia stricta, Chromolaena odorata* and *Mimosa pigra*) are listed amongst 100 of the world's worst invasive alien species (Invasive Species Specialist Group 2013).

Data collection

The data were collected for the MOSAIK project (Monitoring Savanna Biodiversity in the Kruger National Park), whose primary objective was to sample plant and animal biodiversity in habitats across KNP (Delabye et al. 2022; Hejda et al. 2022; Čuda et al. 2024). To this purpose, we established triplets of 50 m × 50 m plots, each triplet including a site: (i) near a perennial river or another permanent



Figure 1. A The Kruger National Park with the location of the 60 sampled sites, separated according to habitat and distributed across the four land systems. The colour of the symbols refers to the habitat and its size indicates the change in the number of alien species recorded in a plot between the first (2019–2020) and second (2024) sampling **B** distribution of the two alien species that exhibited the most pronounced spread during the study period.

source of water such as a dam or pool (the criterion being water present all year round), (ii) near a seasonal river, defined as a river or stream where water is only present in the rainy season and (iii) on a dry crest at least 5 km from any source of water (Fig. 1). The plots within each triplet were selected to capture the different habitats in a similar landscape context within a reasonable distance of $\sim 7-13$ km amongst plots. There were 20 triplets distributed to cover the four land systems in KNP (defined based on the association between geology, terrain morphology, soils and woody vegetation; Venter 1990) with five triplets in each, giving a total of 60 plots (Fig. 1). Consequently, each of the three habitats was sampled with 20 plots and each of the two bedrock (granite, basalt) types with 30 plots.

During two rainy seasons, 16 January to 4 February 2019 and 17 January to 3 February 2020 (further termed 'first sampling'; see Hejda et al. 2022 for more details), all vascular plant species were recorded in each 2500 m² plot and their abundance estimated visually using the Braun-Blanquet cover-abundance seven-grade scale (Mueller-Dombois and Ellenberg 1974). From the data, we identified species that are alien to South Africa and considered introduced by humans to regions outside their native range (see Pyšek et al. 2004; Essl et al. 2018 for definitions). To classify alien species as naturalised (forming self-sustainable populations in the wild) or invasive (a subgroup of naturalised species rapidly spreading in the invaded area), we followed the definition proposed by Richardson et al. (2000) and Blackburn et al. (2011). This classification of species status was based on a recent catalogue of alien plants in KNP (Foxcroft et al. 2023).

On 9–21 March 2024 (further termed 'second sampling'), the same plots were surveyed with a focus on all alien species, not only those recorded in 2019–2020, but also those that arrived since the first sampling. Given the sampling dates in the first period, 33 plots were surveyed after five years and the remaining 27 after four years (see Hejda et al. 2022). The covers of all alien species recorded were very low and did not exceed degree 1 of the Braun-Blanquet scale (Mueller-Dombois and Ellenberg 1974), which corresponds to 1–5% of the plot cover; the covers of species that qualified for this assessment mostly approached even the lower threshold of this degree percentage value. Therefore, the analyses focused on species' presence and absence in the plots rather than their abundance proxied by cover.

Statistical analysis

The differences in the numbers of aliens (frequencies of occurrences in the sampled plots) were tested by generalised mixed-effect models (GLMM), with the triplet identity set as a random effect and the three plots within a triplet considered as pseudoreplicates: m1 <- glmer(number of alien species ~ sampling time*habitat type +(1|triplet), family=poisson). We also ran a GLMM model that included "land systems" and "triplets" (nested in land systems) as random effects: glmer(number of species ~ sampling time*habitat +(1|landsytem)+(1|triplet),family=poisson). This model provided results very similar to the GLMM with only "triplets", which yielded lower AIC, indicating better parsimony (424.5 compared to 425.9 of the model that included land systems). Therefore, we only present the results of the GLMM model with triplets. The GLMM models were created using the package "lme4" of the R statistical software (Bates et al. 2015). The significance of individual terms (sampling time: first sampling in 2019–2020 vs. second in 2024; habitat type: perennial river, seasonal river, crest; sampling time:habitat type interaction) were tested by likelihood ratio tests, where non-significant terms (p > 0.05) were deleted. The differences between individual levels of habitat type were tested by the Tukey post-hoc method, using the R package "emmeans" (Lenth 2024). The accuracy of the parsimonious model was checked by inspecting the normality of residuals using the Shapiro-Wilk normality tests.

The numbers of persisting, newly emerging and disappearing species (in comparison with the first sampling) in individual habitats (perennial rivers, seasonal rivers, crests) were tested using log-linear models of the R software. Similar to the GLMM models with the number of alien plant species as a response variable, the species persistence category (persisting, newly emerging, disappearing) and habitat were predictors. The significance of individual terms was tested by likelihood ratio tests and the differences between the levels of factor predictor (persistence category: persisting species, newly-emerging species, disappearing species; habitat type: perennial rivers, seasonal rivers, crests) were tested by Tukey post-hoc comparisons using the package "emmeans". The differences in the frequencies of occurrences of individual species between the two samplings were tested using chi-square tests, by comparing the total numbers of plots, in which the species was recorded in 2019–2020 versus in 2024, with the aim to find out which alien species decreased or increased their total frequency of occurrence, regardless of the three different habitats.

A direct gradient analysis (CCA) was used to test the overall differences in plant species composition between the first and second sampling. A split-plot sampling scheme was used to reflect the hierarchical arrangement of plots in triplets. The triplets were set as whole-plots and the plots within the triplets were the split-plots. Both whole-plots and split-plots were permuted freely, with 499 permutations.

Results

Numbers and frequencies of alien species

In the first sampling period, 23 alien species were recorded and, in the second sampling, there were 20. In total, 25 alien species were recorded over the five-year study period. Some species data presented here differ slightly from those shown in the previous paper (Pyšek et al. 2020a). The reasons for this discrepancy are: (i) revisiting the native status in uncertain cases (e.g. *Boerhavia diffusa*, *Mollugo nudicaulis* and *Schkuhria pinnata* are now considered alien, while *Litogyne gariepina* and *Melanthera scandens* were reconsidered as native), (ii) re-identification of herbarium specimens of rare species, which yielded alien *Amaranthus standleyanus*, *Conyza bonariensis* and *Sesbania bispinosa* that are now included in the list (Table 1).

The frequencies of alien species, expressed as the total number of species-plot records were 118 in 2019–2020 and 122 in 2024 (Table 1) and differed significantly amongst individual habitats ($\chi^2 = 97.929$, DF = 2, p < 0.001). The Tukey tests showed that perennial rivers harboured more aliens than either seasonal rivers or crests (both p < 0.001), but the difference between seasonal rivers and crests was not significant. Further, there was a marginally significant interaction between the sampling time and habitat ($\chi^2 = 4.744$, DF = 2, p = 0.093), indicating that the distribution of aliens in individual habitats (perennial rivers, seasonal rivers, crests) differed between the first and second sampling. In particular, the crests exhibited even fewer species-plot records in 2024 than in the first sampling in 2019–2020 (Table 1).

Persistence of alien species

The distribution of species amongst the persistence categories (persisting, newly emerging, disappearing) differed ($\chi^2 = 27.467$, DF = 2, p < 0.001; Table 2). There were fewer persisting species than newly-emerging alien species and disappearing alien species (both p < 0.001 in Tukey post-hoc comparisons). There was also a significant interaction between the habitat type and persistence category ($\chi^2 = 15.486$, DF = 4, p = 0.004), indicating that the frequency of species in persistence categories differed according to habitats. This is most likely due to lower persistence of aliens at seasonal rivers and crests (Table 2).

The persistence, emergence and disappearance of individual species are presented in Table 1. The percentage persistence (defined as % of plots in which a taxon was present in both the first and second samplings; Table 1) ranged from very high values (*Acanthospermum hispidum* 80%, *Althernanthera pungens* 75%, *Gomphrena celosioides* 57%) to very low (*Mollugo nudicaulis* 7%, *Bidens bipinnata* 17%) to complete disappearance (e.g. *Conyza bonariensis, Achyranthes aspera*). In the latter **Table 1.** The occurrence of alien species in the Kruger National Park, recorded over two time periods. Species presented in bold significantly differed in their frequency in 60 plots between the two periods and the statistics of the difference are shown. Species marked with an asterisk are not listed in Pyšek et al. (2020) (although they were recorded in 2019–2020), because of reconsideration of their status or re-determination of the botanical material (see text for details). Life history: a – annual herb, p – perennial herb, ss – subshrub, s – shrub. The last column indicates the number of plots in which the taxon persisted (P), newly emerged (E) and from which it disappeared (D) between the first and second sampling. Status in the recent catalogue of alien plants in KNP (Foxcroft et al. 2023) is (St column): n = naturalised, i = invasive, u = unknown, – = not listed.

Taxon	St	Family	Life history	Origin	2019–2020	2024	χ^2	p-value	P-E-D
Acanthospermum hispidum	n	Asteraceae	a	tropical America	5	9	_	_	4-5-1
Achyranthes aspera	n	Amaranthaceae	р	Mediterranean	2	10	6.23	0.013	0-10-2
Alternanthera pungens	n	Amaranthaceae	р	tropical America	8	13	-	_	6-7-2
Amaranthus standleyanus*	-	Amaranthaceae	a	S America	1	0	-	_	0-0-1
Argemone ochroleuca	n	Papaveraceae	a	N America	1	0	-	_	0-0-1
Bidens bipinnata	n	Asteraceae	а	Asia, N America	6	9	-	_	1-8-5
Bidens biternata	n	Asteraceae	a	E Asia (Himalayas)	10	10	_	_	3-7-7
Boerhavia diffusa*	u	Nyctaginaceae	a, p	tropics and subtropics	8	7	-	_	2-5-6
Chenopodium album agg.	-	Amaranthaceae	а	Eurasia	1	0	-	_	0-0-1
Conyza bonariensis*	n	Asteraceae	а	C and S America	2	11	6.23	0.013	0-11-2
Datura inoxia	i	Solanaceae	a, p, ss	N America	1	0	-	_	0-0-1
Gomphrena celosioides	n	Amaranthaceae	a, p	S tropical America	7	11	-	_	4-7-3
Malvastrum coromandelianum	n	Malvaceae	a, p, ss	tropical to subtropical America	10	9	-	_	4-5-6
Mollugo nudicaulis*	-	Molluginaceae	a	unclear	14	3	8.90	0.003	1-2-13
Opuntia ficus-indica	n	Cactaceae	р	C America	0	2	_	_	0-2-0
Opuntia stricta	i	Cactaceae	р	N America	3	3	_	_	1-2-2
Parthenium hysterophorus	i	Asteraceae	a	N America	8	7	-	_	3-4-5
Portulaca oleracea	n	Portulacaceae	a	Eurasia	4	1	-	_	1-0-3
Senna septentrionalis	n	Fabaceae	s	C America	0	3	3.00	0.083	3-0-0
Schkuhria pinnata*	u	Asteraceae	a	S America	9	7	-	_	4-3-5
Sesbania bispinosa*	n	Fabaceae	a	tropical Asia and Africa	1	1	-	_	0-1-1
Tridax procumbens	n	Asteraceae	a, p	C America	11	2	6.23	0.013	0-2-11
Verbesina encelioides	n	Asteraceae	a	S America	1	0	-	_	0-0-1
Xanthium strumarium	i	Asteraceae	a	N America	3	3	-	_	0-3-3
Zinnia peruviana	i	Asteraceae	a	America	2	1	-	_	1-0-1
Number of species					23	20			
Sum of occurrences									
Total					118	122			
Perennial rivers					70	84			
Seasonal rivers					25	26			
Crests					23	12			

Table 2. Persistence of alien species in particular habitats between first (2019–2020) and second (2024) sampling. The numbers are species-plot records of all aliens in each category.

Persistence category	Perennial	Seasonal	Crest	Total
Persisting	29	4	2	35
Newly emerging	55	22	10	87
Disappearing	41	21	21	83

two species, the disappearance from a few plots was compensated by new emergence in many plots at the second sampling. The percentage emergence (% of the total number plots in which the species was present only at the second sampling; Table 1) ranged from 43% to 100%, indicating that, for most species, their current frequency is mainly due to new records (Table 1).

Changes in frequencies of individual alien species

Of the 26 species recorded in both periods, only six (23.1%) have significantly changed their frequency (Table 1). Three species that increased are *Achyranthes aspera*, *Conyza bonariensis* (both from 3% to 18% of plots) and *Senna septentrionalis* (not present in 2019–2024, in 3% of plots in 2024), while *Mollugo nudicaulis* (from 26% to 5% of plots) and *Tridax procumbens* (from 18% to 3%) decreased (Fig. 2). The changes in frequencies of these species within habitats are shown in Suppl. material 1.

The direct gradient analysis (CCA) with binary data on the presence/absence of individual alien species as responses revealed significant compositional differences between the first sampling and second sampling (p = 0.004) because, as described above, some aliens considerably increased their frequencies, while others declined (Suppl. material 2).





Discussion

Stable alien flora composed of fluctuating species

Although we found differences in species composition of the alien flora in KNP as recorded in 2019–2020 vs. 2024, the total number of alien plant species has not changed over the 4–5-year period of the study. There was a decrease in the total number of aliens recorded across all plots, from 23 to 20, but this difference was not significant. However, individual alien species tend to fluctuate, as revealed by the low persistence of some species, along with the numbers of those newly emerging or disappearing. These data illustrate that low persistence in sites once colonised is not a constraint to successful invasion at the scale of the whole Park. Moreover, it needs to be emphasized that some globally noxious invaders continue to persist in KNP, such as *Parthenium hysterophorus* (Foxcroft et al. 2024) and *Opuntia stricta* (Novoa et al. 2021); the first species is listed amongst the invasive species of European Union concern (Brundu et al. 2022) and the second amongst IUCN 100 worst species (Invasive Species Specialist Group 2013).

Concerning individual species, *Achyranthes aspera*, *Conyza bonariensis* and *Senna septentrionalis* increased their frequency most remarkably; *A. aspera* invaded near perennial and seasonal rivers, *C. bonariensis* mainly near perennial rivers and on crests and *Senna septentrionalis* invaded near perennial rivers. The opposite trend, i.e. being markedly less frequent during recent sampling, was found for *Tridax procumbens*, especially near perennial rivers and on crests.

Determining native or invasive status and distinguishing morphologically similar plant species is a challenge for field-based research like that presented here. An example of the former would be *Achyranthes aspera*, which is considered native to many parts of northern, western and eastern Africa, but it is very likely alien to South Africa (van der Walt 2009, https://www.botany.cz). The situation is further complicated by the treatment of this species at the infraspecific level. In KNP, the *A. aspera* var. *sicula* is considered an alien taxon (van der Walt 2009). However, at the species level, *A. sicula* is sometimes referred to as a synonym of *A. aspera*. The genus *Boerhavia* is an example of the latter issue – *B. diffusa* is considered alien to the broader region around KNP (Pooley 1998), while *B. repens* is regarded as native (van der Walt 2009). However, it is very difficult to distinguish these two species in a sterile state. While we consider it fair to admit these uncertainties we encountered, we acknowledge that they do not affect the results reported here that focus on the overall pattern in species richness over time.

Comparison with the most recent catalogue of alien plants in KNP (Foxcroft et al. 2023) revealed that five species recorded in our plots are considered invasive at the whole-park scale, 14 are naturalised and two (*Boerhavia diffusa*, *Schkuhria pinnata*) are listed with unknown status (Table 1). Of the latter category, both species were relatively common in the study plots and their occurrence remained constant over time (seven plots, i.e. 12%) – this might be a reason to reconsider their status as naturalised. Three taxa (*Amaranthus standleyanus*, *Chenopodium album* agg. and *Conyza bonariensis*) represent additions to the alien flora of KNP. *Mollugo nudicaulis* is not listed as alien by Foxcroft et al. (2023), who considered it extralimital. The information on the origin of this species varies; in our treatment, we considered it alien, following the local flora of the Limpopo Valley (van der Walt 2009).

The role of habitat

The distribution of alien species in the three habitats studied, i.e. perennial rivers, seasonal rivers and crests, reveals a similar pattern as in 2019–2020, with perennial rivers showing higher numbers of aliens compared to either seasonal rivers or crests (Pyšek et al. 2020a). Interestingly, we found a marginally significant interaction between the effects of the sampling period and habitat, suggesting that the distribution of aliens in habitats between the two sampling dates differs. It appears that the crests have become even poorer in aliens since 2019–2020.

A similar result documenting the lower capacity of dry sites to harbour alien species (Rejmánek 1989; Milton and Dean 2010; Pyšek et al. 2017) is that the numbers of species that persisted in the same plot at crests from 2019–2020 to 2024 were lower than those of newly-recorded species and those that disappeared. Species persistence was higher near perennial rivers than near either seasonal rivers or on crests.

It is rather speculative to explain these trends, based on the relatively short time between the two samplings. One factor that may play a role is the warming climate that is generally unsuitable for plant invasion in arid areas (Pyšek et al. 2017) and, for KNP specifically, it has been shown that high temperatures constrain the plant species richness in general (Hejda et al. 2022).

Even the losers: rapidly emerging invasion threats

Despite relatively stable invasion-related characteristics of the whole flora across the habitats studied, the rapid spread observed for some species, namely Conyza bonariensis (Asteraceae), indicates that future invaders may suddenly recruit from relatively small and inconspicuous populations that were persisting in the landscape for a long time. Conyza bonariensis was first recorded in KNP in 1952 (Foxcroft et al. 2023), but remained rare for decades and, thus, was considered unsuccessful as an invader. Currently, this species has well-established populations in the southern part of the KNP (Fig. 1B), forming dominant stands in many places (Fig. 3). As one of a few spreading aliens, it is not restricted to river plots -36% of its occurrences are on crests (Suppl. material 1). Its spread may become a serious conservation problem because crests often harbour rare species (Hejda et al. 2022). The rapid recent spread of this species is emphasised by it not being included amongst invasive taxa even in the most recent edition of the catalogue of alien plants in KNP (Foxcroft et al. 2023). Currently, its populations are naturalised in KNP and starting to spread. Therefore, it can already be considered invasive by standard ecological definitions (Richardson et al. 2000; Blackburn et al. 2011), but to confirm this status with certainty, a longer observation period is needed. Seeds of this species are easily dispersed by wind and C. bonariensis could, therefore, invade large areas of savanna in KNP at the expense of resident vegetation and native species. Conyza bonariensis is a common weed in dryland minimum tillage farming systems in Australia and the Mediterranean, with seeds germinating best from shallow burial and in lighter soils (Wu et al. 2007). Conditions in savannas are likely similar to those in minimum tillage fields, with C. bonariensis occupying patches of bare soil disturbed by large herbivores. The species was documented to be resistant to glyphosate in the Mediterranean, South America and South Africa (Dinelli et al. 2008), which makes it difficult to manage. The invasion potential of


Figure 3. *Conyza bonariensis* is an alien species that was quite rare in studied plots in 2019–2024, but its presence increased more than five times (2 vs. 11 records) and it has become widespread and locally abundant beyond the surveyed plots in some parts of KNP. The upper image shows a stand near the N'waswitsontso River, the bottom image a detail of the inflorescence.

C. bonariensis, a species originating from South America, is confirmed by its ranking amongst the naturalised alien species of the world, based on the analysis of the GloNAF database (van Kleunen et al. 2015, 2019). This species has been recorded as naturalised in 227 regions of the world (out of the 844 assessed), including 38 regions in Africa, which ranks it as the 31^{st} most widely distributed alien naturalised species of the ~ 14,000 registered (Pyšek et al. 2017).

In general terms, extreme environments, such as arid ones, may harbour relatively few invasive species, but these are often strong invaders (e.g. Clarke et al. 2005; Kumar and Mathur 2014) and their impact on biodiversity can be severe because dry habitats often host rare species that are threatened by the invader (e.g. Gaertner et al. 2009; Fried et al. 2014).

Achyranthes aspera (Amaranthaceae) is not as widespread and dominant beyond the surveyed plots as *C. bonariensis*, but it is not restricted to a specific part of KNP (Fig. 1B). It is listed as naturalised in the recent catalogue of alien plants in KNP (Foxcroft et al. 2023). The fruit of this species has spiny bracts that adhere to animal skin and is thus effectively spread over a long distance (Bullock and Primack 1977). Spread by animals may be a reason for the strong affiliation of this species with rivers (91% of occurrences are along rivers; Suppl. material 1). Reported as naturalised from 160 regions of the world, 52 of which are located in Africa (Pyšek et al. 2017), this species is another candidate for a future problematic invader.

Longitudinal field data from permanent plots are the best way to precisely record changes in the performance and distribution of alien species, for which rapid dynamics are typical. The relatively short period assessed in the current paper indicates that changes in the performance of individual alien and invasive species may occur relatively quickly. To determine the consistency of observed trends and whether the increases or decreases of some species are just fluctuations over time, it is advisable to collect such data over a more extended observation period. More generally, alien species are linked to biodiversity change, but the extent to which they are associated with the reshaping of ecological communities is not well understood; repeated sampling of plots where alien plants were recorded in the past, such as the BioTime database (Dornelas et al. 2018; Knollová et al. 2024) has potential to provide insights into how alien species affect plant community dynamics. A study using data from repeated surveys found that, even in communities where alien species were typically rare, their presence was associated with an increase in the average rate of compositional change, mainly due to species replacement (Kortz et al. 2023).

Our paper shows that even small populations of alien species that fluctuate in their abundance and shift their distribution across savanna habitats may exhibit considerable dynamics over a short period of time and present threat to savanna biodiversity. These results can provide justification for managers in protected areas to argue for funding to repeatedly survey alien species, their distributions and impacts.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

PP initiated the idea, MH, JČ and PP collected the data, MH analysed the data, PP and MH wrote the first draft of the manuscript, JČ, KP and MH prepared the figures, all authors discussed the results, contributed to editing and writing.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Number of records of species whose frequency has significantly changed between the first and second sampling, shown separately for particular habitats

Authors: Petr Pyšek, Jan Čuda, Llewellyn C. Foxcroft, Klára Pyšková, Martin Hejda Data type: docx

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Supplementary material 2

Direct ordination (CCA) plot showing the overall differences in the distribution of aliens between the first sampling in 2019–2020 and resampling in 2024 (p = 0.002)

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- Explanation note: The plot shows that some aliens, like Amaranthus standleyanus, were more frequent in 2019–2020, while others, like Conyza bonariensis, were more frequent in 2024. AcanHisp = Acanthospermum hispidum, AchrAspr = Achyranthes aspera, AltPung = Althernanthera pungens, AmarStan = Amaranthus standleyanus, ArgmOchr = Argemone ochroleuca, BidnBipn = Bidens bipinnata, BidnBitr = B. biternata, BoerDiff = Boerhavia diffusa, ConzBonr = Conyza bonariensis, DatrInox = Datura inoxia, GompCels = Gomphrena celosioides, ChnAlbAg = Chenopodium album agg., MalvCorm = Malvastrum coromandelianum, MollNud = Mollugo nudicaulis, OpunFics = Opuntia ficus-indica, OpunStrc = Opuntia stricta, PartHyst = Parthenium hysterophorus, PortOler = Portulacca oleracea, SchkPinn = Schkuhria pinnata, SennSept = Senna septentrionalis, SesbBisp = Sesbania bispinosa, TridProc = Tridax procumbens, XantStrm = Xanthium strumarium, VerbEnce = Verbesina encelioides, ZinnPerv = Zinnia peruviana.
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Research Article

Invaders break assembly rules to beat the natives: how cheatgrass cheats

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Abstract

Understanding how some introduced plants achieve invasive status while most simply become naturalized is a fundamental question in invasion ecology. Traditional approaches comparing native and introduced plants have linked ruderal traits such as annual life history, high fecundity, and rapid growth rates to invasiveness. However, they do not explain why other introduced species bearing similar traits fail to become invasive, possibly because generic comparisons ignore local processes that drive community assembly. Herein, we contrasted native and introduced annuals in the context of local successional processes to elucidate how introduced annual bromes like cheatgrass (Bromus tectorum) overtake perennial grasslands in the intermountain western United States. We created disturbed plots and seeded them first with annuals representing natives, naturalized species, or invasive bromes. We then seeded plots with native perennial community dominants to examine how the different annuals influenced succession. Native annuals established transient populations that facilitated perennial establishment compared to unseeded controls, enabling the shift to perennial dominance. Naturalized annuals mirrored the natives, but invasive annuals maintained robust populations at high biomass that inhibited perennial establishment and impeded succession. Mechanistically, invasive annuals reduced soil moisture and elevated plant biomass, litter, and soil N. However, only litter abundance correlated with perennial seedling recruitment across treatments. Overall, litter showed a unimodal relationship wherein lower litter abundance associated with native and naturalized annuals appeared to facilitate perennial seedling establishment while higher litter levels generated by invasives appeared to suppress perennial establishment and inhibit succession. Additional experiments provided little support for the roles of pathogen spill-over or plant-soil feedbacks favoring the introduced bromes. The domination of perennial grasslands by annual bromes may be driven by litter buildup that allows these introduced plants to break local succession rules by acting as both early seral and climax species. Traits like litter accumulation may strongly influence invasion outcomes but are indistinguishable using trait comparisons lacking community context.

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Key words: Annual bromes, community assembly, ecosystem engineering, extended phenotype, grassfire cycle, invasive plants, litter, naturalized plants, pathogen spillover, plant-soil feedback, succession

Introduction

Some introduced species cause inordinate ecological and economic damage (Mack et al. 2000; Vilà et al. 2011). However, most fail to establish or merely become naturalized with little measurable impact on their new environments (Williamson and Fitter

1996; Pearson et al. 2016). Identifying those unique traits that allow some introduced species to achieve pest status, i.e., become invasive, remains the ultimate objective of invasion ecology (Wallace 1881; Sax and Brown 2000). Traditional approaches to this problem have focused on comparing native and introduced plants to identify traits linked to invasiveness (Van Kleunen et al. 2010; Gallagher et al. 2015; Moravcová et al. 2015). However, these methods compare traits among species without reference to the local community assembly rules that determine the linkage between traits and fitness outcomes (Pearson et al. 2018a). While such studies have broadly linked invasiveness to ruderal strategies such as high fecundity and rapid growth (Pysek and Richardson 2007; Van Kleunen et al. 2010; Jelbert et al. 2015; Moravcová et al. 2015), they do not explain why many introduced plants bearing similar traits simply become naturalized (non-invasive) community members. Comparing native and introduced species in the context of local community assembly processes may help to explain how some introduced species become invasive while others do not (Pearson et al. 2018a), despite sharing superficially similar traits. Moreover, given the strong linkages between ruderal traits, disturbance, and plant invasions (Davis et al. 2000; Pysek and Richardson 2007; Van Kleunen et al. 2010; Jelbert et al. 2015; Moravcová et al. 2015, Pearson et al. 2018b), contrasting native and introduced ruderals in the context of local successional trajectories could prove fruitful.

Succession theory offers the longest-standing framework for explaining plant community assembly (Cowles 1899). Several derivations of succession theory exist (e.g., Cowles 1899; Clements 1916; Gleason 1926; Odum 1969; Connoll and Slatyer 1977) with some arguing for a more deterministic Clementsian model (Clements 1916) and others for a more stochastic Gleasonian (Gleason 1926) perspective. Nonetheless, a common theme is that, following disturbance, plant communities assemble along identifiable stages, known as seres, that culminate in a climax community state reflective of local edaphic and climatic conditions (summarized in Lomolino et al. 2010: 133-134). Within this framework, earlier seres tend to be dominated by pioneering or ruderal species bearing traits such as high fecundity, high vagility, and rapid growth that allow them to quickly exploit the high-resource, low-competition conditions that commonly follow disturbances. Over time, early seral species give way to slower, more competitive or stress tolerant species that may arrive later due to lower fecundity and vagility, but are better adapted to low-resource, high-competition conditions occurring late in succession. The most tolerant and well-adapted of these late seral species comprise a self-perpetuating community referred to as the climax state, which serves as the foundation for classifying communities based on predictable associations with abiotic conditions (e.g., Daubenmire 1966; Pfister and Arno 1980). Yet, how introduced plants may fit into or disrupt these natural successional processes remains understudied (but see Tognetti et al. 2010).

If we apply this succession framework as a benchmark for understanding the rules by which native plant communities assemble (*sensu* Pearson et al. 2018a), we may begin to reveal when and how some introduced plants break the rules to gain advantage over natives in the course of succession. Since many introduced plants exhibit r-selected traits associated with early-seral or ruderal strategies such as high fecundity and rapid growth (Pysek and Richardson 2007; Van Kleunen et al. 2010; Jelbert et al. 2015; Moracová et al. 2015), we might expect such introduced plants to exploit early successional stages to become abundant immediately following disturbance (e.g., Davis et al. 2000; Jauni et al. 2015; Pearson et al. 2018b). Following the rules of succession described above, these species should give way to lat-

er seral species as seen in native communities (see Lomolino et al. 2010). However, the fact that some introduced annuals come to dominate perennial plant communities where native annuals historically played early seral roles (Mack 1981; Prober et al. 2005; Brooks et al. 2016) suggests that such invaders may somehow break the rules of succession by acting both as early seral species that thrive post disturbance and as climax species that maintain dominance thereafter. Yet, most introduced plants do not overtake native communities (Simberloff 1981; Williamson and Fitter 1996; Ortega and Pearson 2005; Pearson et al. 2016), suggesting that most introduced annuals simply follow the rules of succession. While these patterns are compelling, experimentation is required to understand how some introduced annuals become problematic pests while others simply naturalize.

Mechanistically, introduced plants can impact communities through a variety of processes (see Catford et al. 2009). One process particularly relevant to succession is the ability of invasive plants to alter abiotic and biotic conditions that affect competitive interactions with natives. For example, plant-soil feedbacks (PSF) defined as species-specific changes in soil properties that influence plant performance (sensu van der Putten et al. 2013) - may facilitate succession if early seral species experience stronger negative feedbacks than later seral species (Kardol et al. 2006; Kulmatiski et al. 2008). Hence, invaders could break successional rules by generating positive feedbacks on themselves or escaping negative feedbacks experienced by other community members (Klironomos 2002; Levine et al. 2006). Invasive introduced plants can also benefit by suppressing soil mutualists (Stinson et al. 2006) or by enhancing pathogens (Zhang et al. 2020) if effects are stronger on native competitors. Invaders can also promote seed pathogens that suppress native competitors via spillover effects (Meyer et al. 2007, 2014; Flory and Clay 2013). From the abiotic PSF standpoint, many invasive plants change soil fertility either through altered associations with soil biota involved in nutrient cycling (McLeod et al. 2016) or via changes in litter quantity or quality (Liao et al. 2008), with potential ramifications for succession (Paschke et al. 2000). Hence, invader-mediated shifts in both abiotic and biotic properties represent a broad category of processes by which invasive introduced plants may break successional rules.

Within western North American grasslands, cheatgrass (Bromus tectorum) and other annual bromes (Bromus spp.) are notorious invaders that have overtaken vast regions of perennial grasslands and shrublands (Mack 1981; Knapp 1996; Ogle et al. 2003; Germino et al. 2016), despite their annual status. Extensive research targeting these species has established that bromes can elevate litter biomass (Knapp 1996; Evans et al. 2001; Lenz et al. 2003; Bansal et al. 2014), alter available soil moisture (Cline et al. 1977; Booth et al. 2003), indirectly suppress native grass emergence through seed pathogen spillover (Beckstead et al. 2010, 2016; Meyer et al. 2014), and directly suppress native seedlings via competition (Humphrey and Schupp 2004; Yelenik and Levine 2010; Parkinson et al. 2013). Annual bromes can also alter N cycling and ammonium and nitrate levels (Evans et al. 2001; Bansal et al. 2014; Stark and Norton 2015; McLeod et al. 2016), and generate PSFs (Perkins and Nowak 2013). These studies provide important insights regarding several mechanisms that might facilitate brome invasion, but to date no study has evaluated these factors within a successional framework to understand how annual bromes might break succession rules to dominate native perennial grasslands.

We explored the above ideas within Intermountain Grasslands of the western United States that are susceptible to takeover by introduced annual brome grasses

(Mack 1981). These native grasslands are dominated by perennial bunchgrasses and forbs in their climax state (Mueggler and Stewart 1980). In this system, natural disturbances sufficient to displace community dominants are followed by flushes of native annuals that rapidly establish and flourish but ultimately give way to the dominant perennial plant community. Within this context, we established a field experiment wherein we created disturbances and initiated succession by seeding plots with either 1) native annuals, 2) naturalized introduced annuals, or 3) invasive introduced annual bromes. We then seeded six species of dominant native perennial grasses and forbs (climax species) into each plot to examine how succession progressed. Despite the fact that all our annuals were ruderal taxa with high fecundity and rapid growth rates, we predicted that 1) native annuals would succumb to perennials, allowing succession to proceed as seen in natural grasslands, 2) naturalized annuals would behave like the natives, similarly allowing succession to proceed, and 3) invasive annuals would suppress the native perennials and maintain dominance by inhibiting succession. To understand how the different ruderal groups influenced successional trajectories, we evaluated their effects on standing biomass, litter, and soil properties. Finally, we complemented the succession experiment with secondary experiments evaluating PSFs and pathogen spillover.

Materials and methods

Field experiment

We established our main experiment within a fenced space at Diettert Gardens on the University of Montana campus, Missoula, MT, USA in 2014 (46.841981, -113.992030). The site reflects abiotic conditions found in the adjacent intermountain bluebunch wheatgrass habitat (Mueggler and Stewart 1980) and was historically dominated by this grassland community. The native plants selected for the experiment represent perennial community dominants and common early seral annuals based on Mueggler and Stewart (1980) and extensive vegetation surveys conducted across these systems (Pearson et al. 2016). For the invasive introduced (hereafter "invasive") species, we chose cheatgrass (*Bromus tectorum*) and Japanese brome (*B. japonicus*) because these species are highly invasive annuals capable of dominating perennial grasslands of western North America (Mack 1981; Knapp 1996; Ogle et al. 2003; Germino et al. 2016). For the naturalized introduced (hereafter "naturalized") species, we selected four common annuals that thrive following disturbances but fail to dominate intermountain grasslands (mean cover $\leq 3\%$ as reported by Pearson et al. 2016).

We prepared the site by watering for two weeks to initiate seed germination and purge the seedbank in August 2015. We then applied Roundup[®] to kill actively growing plants in September. In October, after the herbicide had degraded, we delineated 100 0.7×0.7 m (0.5-m²) plots with 0.8-m spacing (separated by weed cloth to maintain plot integrity) in a rectangular array and simulated disturbance in each plot by digging the soil to a depth of ~15 cm and removing plant biomass. We then sowed each plot with 1000 seeds of one of nine focal annual species, with ten replicate plots per species randomly assigned to represent the following treatments: 1) natives (*Collinsia parviflora, Clarkia pulchella*, or *Plantago patagonica*), 2) naturalized taxa (*Alyssum alyssoides, Arenaria serpyllifolia, Filago arvensis*, or *Veronica verna*), and 3) invasives (*Bromus japonicus* or *B. tectorum*). The remaining 10

plots were left unseeded to represent controls. In September 2016, after one growing season, we seeded all plots with three species of native perennial grasses (*Festuca idahoensis*, *Koeleria macrantha*, and *Pseudoroegneria spicata*) and three species of native perennial forbs (*Achillea millefolium*, *Balsamorhiza sagittata*, and *Lupinus sericeus*), all representing climax species that dominate undisturbed grasslands in this system (seeding rates were 100, 150, and 200 seeds per plot for large, medium, and small-seeded species, respectively, to reflect natural variation in fecundity [*sensu* Maron et al. 2012]; Suppl. material 1: table S1). All seeding and sampling targeted the center 0.5×0.5 m of each plot, allowing a 0.1 m wide buffer zone on all sides. Non-study species were weeded to < 10% cover in all plots each year, and focal invasives were completely weeded from treatments where not sown to prevent them from overtaking other treatments. Other focal annuals were allowed to colonize plots where they were not sown because it was not possible to remove them before they went to seed without disturbing perennial seedlings.

In the first growing season after seeding perennials, we documented recruitment by counting seedlings in each plot twice during the main germination window, May-June 2017. We carefully removed perennial seedlings after the final count and then reseeded the plots with the same species in September 2017, following the prior year's methods. This approach allowed us to quantify perennial recruitment over two growing seasons. In spring 2018, we counted perennial seedlings as done previously, but this time we left plants to grow. To represent recruitment per plot and year in analyses, we took the maximum count across the two surveys for each sown perennial species and summed these across species. Late in the growing season of 2019, we harvested all plots for aboveground biomass. Biomass of live focal annuals, sown perennials and litter (dead material from past years) was separated and dried for 48 hours at 62 °C before weighing.

To track populations of individual focal annuals in the years preceding harvest, 2016–2018, we visually estimated cover of both seeded and colonizing species in each plot (to the nearest 1% if < 10%, and nearest 5% if > 10%) aided by a frame marked in 1% increments. For examination of factors influencing perennial recruitment, we considered total focal annual cover summed across all functionally similar species per plot. For native, naturalized, and control treatments, this metric included focal native and naturalized annuals directly sown in the plots and those colonizing from other plots, as justified by comparison of perennial recruitment among plots established with these taxa (Suppl. material 1: fig. S1). Similarly, we treated the invasives B. japonicus and B. tectorum as a species complex given that they intermixed to some degree and were difficult to distinguish until late season (i.e., after cover surveys). Biomass harvested in 2019 showed that plots were dominated by the original sown Bromus in 70% of n = 20 cases, and perennial recruitment did not differ between plots sown with the two species (Suppl. material 1: fig. S1). We followed these same pooling conventions for consideration of focal annual biomass. We also measured litter cover in each year prior to harvest, 2016–2018. Cover estimation was repeated in 2019 prior to harvest for comparison to biomass.

To understand how focal annuals might affect abiotic conditions, we measured soil properties in each study plot in 2018. We extracted four soil cores (2.5 cm diameter x 10 cm deep) from each plot in mid-May. Soil cores were pooled by plot and sieved through a 2 mm sieve before lab analyses. We used a subset of each fresh soil sample to quantify available N (NO₃⁻ and NH₄⁺) via KCl extraction (Hart et al. 1994) followed by colorimetric analyses on a Synergy 2 Microplate Reader (BioTek,

Winooski, VT, USA) according to Weatherburn (1967) and Doane and Horwáth (2003). The remainder of each soil sample was air dried and sent to Ward Laboratories (Kearney, NE, USA) for measurements of SOM (LOI%), pH (water), P_{Merlich}, K, S, Zn, Fe, Mn, Cu, Ca, Mg, Na, and CEC. We sampled soil moisture (volumetric water content) at 4 cm depth weekly from May-June using a handheld probe (Field Scout[®] TDR-100; Spectrum Technologies, Inc., Plainfield, Illinois, USA) at two locations near the center of each plot and used the mean value per plot in analyses.

The invasive annuals B. tectorum and B. japonicus host the seed pathogen Pyrenophora semeniperda which can spillover onto and suppress germination of native grasses (Beckstead et al. 2010, 2016; Meyer et al. 2014). To evaluate whether this pathogen and/or other generalist seed pathogens like Fusarium (Meyer et al. 2014, 2016) played a role in our experimental results, we carefully tracked fates of marked seeds of *P. spicata* in a subset of field plots. This effort focused on *P. spicata* 1) to render the experiment manageable, 2) because this species is known to be susceptible to these pathogens, and 3) it is the dominant perennial grass in our system. To track the fate of *P. spicata* seeds, we glued individual seeds to the base of a wooden toothpick with Elmer's School Glue® and placed it in the ground so that the seed was buried in the soil (after Meyer et al. 2014). This was done for 20 seeds per plot in all *B. tectorum* and *B. japonicus* plots (n = 10 each), all control plots (n = 10), and half of the plots seeded with each native and naturalized species, respectively (n = 35). Seeds were planted in the center of each plot in a 5×4 grid with 10 cm spacing in early October 2018. In November 2018, we scored fall emergence and in early May 2019 we scored survival of fall seedlings and emergence of spring seedlings. We could not follow survival of marked seedlings beyond this point because the sample size of those remaining was limited for invasive plots (80% of plots had < 3 seedlings; range 0-5 seedlings). We recovered marked seeds with no evidence of emergence in field surveys and examined them under a dissecting scope for signs of pathogen attack including typical "black fingers of death" structures associated with Pyrenophora and infection cushions associated with Fusarium (Meyer et al. 2016).

Greenhouse PSF experiments

We initiated two PSF experiments in the greenhouse. The first experiment was designed to evaluate feedbacks of individual focal annuals on themselves and the second was designed to assay feedbacks from the focal invasive, *B. tectorum*, on the native perennials sown in our field experiment. This second experiment focused on *B. tectorum* to simplify logistics. Because we were interested in both abiotic and biotic PSFs, we followed the approach outlined in Castle et al. (2016) where soil was first trained by relevant species in "round 1" of the experiment. Shoot biomass was then harvested, and soil was sieved (10 mm) to remove most roots, placed back in the pot, and seeded with species for "round 2."

In round 1 of the first experiment, each focal annual species sown in the field experiment was grown for three months (Nov-Jan) in 650 ml pots pots filled to approximately 500 ml with sieved (3 mm) soil collected to 10 cm deep from the experimental site and mixed with heat-treated sand and Turface (2:1:1, v:v:v). In round 2 of this experiment, seeds of the same species were planted in conspecific-trained soil (8 replicates per species in each round of the experiment for n = 72 pots per round). In both rounds, we grew three plants per plot after weeding out extra plants from the initial seeding effort and watered with tap water as needed. At the midpoint of

each round, all pots received 20 mL of a 0.5 g/L 20-2-20 (N-P-K, Peters Professional fertilizer, JR Peters, Inc., Allentown, PA, USA) solution to address nutrient limitation indicated by yellowing of leaves. At the end of each round, we harvested shoots, dried them for 48 hours at 62 °C, and weighed them. PSFs in this experiment were calculated by comparing biomass between rounds 1 and 2. To address possible differences in environmental conditions between rounds, we also grew each species in an inert medium (1:1 mix of Black Gold® Seedling Mix and Miracle Gro® Seed Starting Potting Mix) during each round (n = 3 replicates/species). In the second experiment, we first trained soils in round 1 by growing either B. tectorum or non-Bromus annuals (each of seven focal species) in pots for three months using the protocol described for the first experiment. In round 2, each of the 6 perennial species sown in our field experiment was grown either in the B. tectorum-trained soils (8 replicates/perennial species for 48 total pots) or in soils trained by non-Bromus annuals (7 replicates/perennial species comprised of 1 pot per non-Bromus species for 42 total pots). A single perennial plant was grown per pot after weeding out extra plants from the initial seeding effort, and plants were watered as needed with tap water. After two months, we harvested shoot biomass as described above. To evaluate PSF in this experiment, we compared biomass of perennials grown in soil trained by B. tectorum vs. by non-Bromus annuals. In both experiments, pots were not root-bound at the end of either round, and the few plants that died were excluded from analyses (n = 4 and n = 8 plants from each experiment, respectively).

Statistical analyses

For our field experiment, we used generalized linear models (GLMs) in SAS (PROC GLIMMIX, SAS Institute 2013) to compare responses (plant/litter abundance metrics, perennial seedling recruitment, marked P. spicata seed/seedling fates, and soil properties) among annual plant treatments, i.e., plots seeded with native, naturalized or invasive annuals, and unseeded controls. For responses measured in multiple years, we ran a separate model for each year. The annual plant treatment was included as a fixed factor in all cases. We did not include focal species identity in models as a random factor given that 1) species within each treatment category functioned similarly (Suppl. material 1: fig. S1) and were allowed to intermix (see above), and 2) we did not intend to extrapolate results to all potential species in each treatment category (as would apply if species was included as a random factor) but rather contrast the behavior of annual bromes to a reasonable representation of native and naturalized annuals, respectively. To further evaluate significant treatment effects (P < 0.05), we tested for post-hoc differences using multiple comparisons wherein the P-value was adjusted for the number of comparisons via the Bonferroni method. Response variables were fit to the most appropriate distribution, as assessed by examining scatterplots of residuals against predicted values (negative binomial for perennial seedling counts; lognormal distribution for biomass and soil P, K, S, Cu, and CEC; beta for proportion of marked P. spicata seeds emerging and seedlings surviving, respectively; and normal for remaining variables). We present least squares means and SEs back-transformed from the scale used in analysis as relevant. For analysis of pathogen presence on unemerged P. spicata seeds, we simplified data to the plot level and tested for differences in attack frequency using Fisher's Exact Test, as more complex analyses were limited by the lack of detections.

We also evaluated the relationship between recruitment of native perennials and conditions in plots to explore potential mechanisms governing succession in our field experiment. To do so, we used GLMs with perennial seedling counts from 2018 (the cohort linked to sampling of soil properties and to final biomass measures in the subsequent year) as the response fit with a negative binomial distribution. Based on observed differences among treatments, we opted to construct separate models for 1) plots established with "non-invasive" annuals (native or naturalized treatments) or eventually colonized by these species (controls), and 2) invasive annual plots. This allowed us to take a simple approach to examining whether mechanisms of perennial establishment might differ for communities dominated by either non-invasive or invasive annuals. Model covariates represented measured biotic and abiotic conditions from 2018, and all were considered in the same multivariate model to isolate the independent influence of each covariate (i.e., when variation attributable to other covariates was accounted for). These covariates were total focal annual cover, litter cover, soil moisture, and soil NH₄⁺ and NO₃⁻ (remaining soil properties showed minimal differences among treatments; see Results). We also included an additional measure of litter abundance, litter biomass, in models because litter properties differed between non-invasive/control and invasive annual plots. For plots dominated by non-invasive species, litter cover captured within-year variation in litter abundance (e.g., litter cover and biomass measured in 2019 were significantly correlated: r = 0.12, P = 0.002), which was largely two-dimensional. However, in invasive plots, old plant material accumulated in three dimensions and litter cover did not suffice to capture variation in litter quantity among plots (e.g., litter cover and biomass measured in 2019 were not significantly correlated: r < 0.1, P = 0.18; see Results). Therefore, in addition to including litter cover in models (as measured in 2018 to align with seedling counts), we also included litter biomass (only measured in 2019). We screened for potential multicollinearity issues by testing whether model covariates were highly correlated in bivariate space (r > 0.9; Tabachnick and Fidell 2007), but these correlations were modest (r < 0.6). While *P*-values tested for significant covariate relationships, we also wanted a means of comparing the relative contribution of each covariate to overall variation in recruitment (akin to an r^2 value). For this purpose, we dropped covariates one at a time from the full model to determine the effect on AICc (Akaike's Information Criterion corrected for small sample sizes), which provides a measure of expected predictive power weighted by the number of model parameters (Bolker et al. 2009).

For the greenhouse experiment designed to compare PSFs of individual focal annuals among native, naturalized and invasive taxa, we treated shoot biomass (mean of three plants per pot) as the response in a generalized linear mixed model in SAS (PROC GLIMMIX, SAS Institute 2013). Fixed factors were round (round 1: growth in untrained soil vs round 2: growth in conspecific-trained soil), annual plant type, and their interaction, and pot was included as a random factor. To test whether environmental conditions differed between rounds for focal annuals grown in an inert medium, we ran a GLM with shoot biomass (mean of three plants per pot and species) as the response and round as a fixed factor. For the experiment designed to test for PSF from the focal invasive, *B. tectorum*, on native perennials, we used a GLM with shoot biomass as the response. Fixed factors in this model were soil type (soil trained by *B. tectorum* vs. by non-*Bromus* annuals), species (each of six perennials from our field experiment), and the soil type x species interaction. For all three analyses of greenhouse biomass responses, we specified a normal distribution based on satisfaction of criteria described above.

Results

Early seral community composition

Focal annuals established rapidly and formed monocultures in plots where sown in the first year after seeding, 2016 (Fig. 1a, Suppl. material 1: table S2). In this year, mean cover per seeded focal species was substantial (\geq 40%) in all seeded treatments, though more than twice as high for invasive compared to native or naturalized annuals (P < 0.001). In 2017, seeded annual species retained their dominance where sown, with similar cover among seeded treatments (Fig. 1b,



Figure 1. Abundance of seeded and colonizing focal annual species by treatment. Given is mean (+ 1 SE) cover per focal species seeded into experimental plots to represent native, naturalized, or invasive annuals, 2016–2018. Plots were disturbed and purged of plants prior to seeding in fall 2015, and control plots were not seeded. Sown native and naturalized annuals were allowed to colonize plots where unseeded, and mean (+ 1 SE) cover per species across this set of colonizing taxa is given for comparison to seeded species cover. Within-year patterns for each variable were evaluated with post-hoc comparisons when the treatment effect was significant (P < 0.05), and means that do not share letters (seeded species: lower case, colonizing species: upper case) are significantly different.

Suppl. material 1: table S2). Focal native and naturalized annuals spread into other plots, except the invasive annual plots, where colonization was negligible (80% lower than other treatments; P < 0.001). In 2018, seeded focal annuals remained a prominent component in all treatments where sown, but only invasive annuals retained monoculture status (Fig. 1c, Suppl. material 1: table S2). By this time, mean cover per seeded annual had diminished to modest levels for native and naturalized species but was more than three times greater for invasives (P < 0.001).

Perennial establishment and succession

Invasive annuals suppressed recruitment of sown native perennials in both 2017 ($F_{3,96} = 15.3$, P < 0.001) and 2018 ($F_{3,96} = 24.1$, P < 0.001; Fig. 2a). In 2017, perennial seedling counts averaged 50% lower in the invasive annual treatment relative to remaining treatments, with no differences among the latter. Despite much higher overall recruitment levels in 2018, the pattern was similar, with 42% fewer seedlings in the invasive treatment compared to native and naturalized treatments, which again did not differ from each other. However, seedling counts in 2018 were also depressed in unseeded controls relative to treatments established with native and naturalized annuals, indicating that these taxa facilitated native perennial recruitment.

This pattern of recruitment translated to marked differences in community composition in 2019 as measured by final plot biomass, with invasive annuals impeding succession toward native perennial dominance (Fig. 2b). Focal annual biomass averaged three times greater in invasive plots relative to native and naturalized plots $(F_{3.96} = 29.9, P < 0.001)$, with no difference between the latter treatments. In contrast, biomass of native perennials was depressed by > 60% in the invasive vs. native and naturalized treatments ($F_{3.96}$ = 5.3, P = 0.002), again with no difference between the latter treatments. Hence, the invasive treatment remained dominated by annuals (invasive annuals had twice the biomass of perennials), while native and naturalized treatments had shifted strongly towards perennials (perennials had > 6 times the biomass of annuals). Focal annual biomass in controls was comparable to levels in native and naturalized treatments but lower than in invasive plots, while native perennial biomass in controls did not differ significantly from other treatments though trended towards the lower end of the continuum (Fig. 2b). As a result, composition in the control treatment was shifted only slightly towards perennials (perennials had 1.4 times the biomass of annuals), with prevalence of the latter limited primarily by poor recruitment relative to native and naturalized treatments in the prior year (Fig. 2a).

Treatment conditions and relationships with perennial recruitment

Both biotic and abiotic conditions differed among treatments, potentially influencing patterns of native perennial recruitment. Total focal annual cover accounted for the combined abundance of those annual species seeded into plots and those sown native and naturalized taxa colonizing from other plots for all but the invasive treatment, where colonization was negligible. Total focal annual cover was greatest in the invasive species treatment in two of three years despite the boost given to remaining treatments by colonizing taxa, and it was generally lowest in the control treatment due to the lack of initial seeding (Fig. 3a, Suppl. material 1: table S3). Total cover of focal annuals did not differ between native and naturalized treatments in any year.



Figure 2. Recruitment of native perennials and final community composition by treatment. Native perennials were seeded into plots representing native, naturalized, or invasive annuals, and controls, and their mean abundance (+ 1 SE) was measured by **a** seedling recruitment in 2017 and 2018, and **b** biomass relative to that of focal annuals at the end of the study in 2019. Sown native and naturalized annuals were allowed to colonize plots where they were not seeded and are included in focal annual biomass in all cases except the invasive treatment, where they were a minor component (Fig. 1). Native perennials were sown in fall 2016, counted and removed in spring 2017, and then sown again in 2017 and counted in 2018 to give two years of recruitment data. Perennial seedlings from 2018 were allowed to grow through the subsequent growing season to evaluate succession. Within-year patterns for each variable were evaluated with post-hoc comparisons when the treatment effect was significant (*P* < 0.05), and means that do not share letters are significantly different.

Litter cover was limited to trace levels (<1%) across treatments in 2016, the first year after annuals were seeded, but in subsequent years, treatments differed markedly (Fig. 3b, Suppl. material 1: table S3). In 2017 and 2018, litter cover was highest in the invasive annual treatment and lowest in controls, matching the overall pattern seen for total cover of focal annuals. Notably, litter biomass in invasive plots was five times that of other treatments by 2019 ($F_{3,96} = 79.1$, P < 0.001; Fig. 3c). This pattern was driven by greater litter depth in invasive plots, as litter cover differed only modestly among treatments by this point (Suppl. material 1: fig. S2).



Figure 3. Total focal annual cover and litter in treatments representing native, naturalized, or invasive annuals, and controls. Given is mean (+ SE) **a** total cover of focal annual species, 2016–2018 **b** litter cover, 2016-2018 and **c** litter biomass at the end of the experiment in 2019. Sown native and naturalized annuals were allowed to colonize plots where they were not seeded and are included in total focal species cover in all cases except the invasive treatment, where they were a minor component (Fig. 1). Photo shows a perennial forb (*Balsamorhiza sagittata*) seedling (circled) emerging from the thick litter layer in an invasive plot. Within-year patterns for each variable were evaluated with post-hoc comparisons when the treatment effect was significant (P < 0.05), and means that do not share letters are significantly different.

Invasive annuals altered soil conditions, as measured in 2018. NH₄⁺ concentration was > 50% higher on average in invasive annual plots relative to those established with native or naturalized annuals ($F_{3,94} = 6.4$, P < 0.001), and NO₃⁻ concentration was on average > 75% higher ($F_{3,93} = 7.8$, P < 0.001; Fig. 4a, b). For both nitrogen measures, control treatments generally fell at intermediate levels, though did not differ significantly from other treatments. Soil moisture was depressed in the invasive treatment, with a decline of 9% relative to naturalized plots and 16% relative to native and control plots ($F_{3,96} = 13.0$, P < 0.001; Fig. 4c). Comparison of additional soil properties showed minimal differences among treatments (Suppl. material 1: table S4).

To consider how these changes in biotic and abiotic conditions might influence patterns of native perennial recruitment, we modeled the relationship between seedling counts from 2018 and measured covariates (Suppl. material 1: table S5). For treatments established with non-invasive annuals (native or naturalized taxa) or eventually colonized by these species (controls), recruitment varied positively with litter cover in the multivariate model that included the full set of covariates (P = 0.025; Fig. 5a). In contrast, for the invasive annual treatment, where litter abundance was relatively high, recruitment correlated negatively with litter biomass (P < 0.001; Fig. 5b), while also correlating negatively with both focal annual cover



Figure 4. Soil conditions in treatments representing native, naturalized, or invasive annuals, and controls. Given is mean (+ SE) **a** available ammonium (NH4⁺) content **b** available nitrate (NO3⁻) content, and **c** soil moisture (volumetric water content); as measured in 2018. For each variable, means that do not share letters are significantly different, as evaluated with post-hoc comparisons when the treatment effect was significant (P < 0.05).

(P = 0.026) and NH₄⁺ (P < 0.001) vs. positively with NO₃⁻ (P = 0.019). When we individually removed covariates from the multivariate model for non-invasive/ control plots, only removal of litter cover reduced model quality (Δ AICc = 2.6). For invasive plots, removal of litter biomass and NH₄⁺ from the multivariate model reduced model quality (Δ AICc = 14.0 and 18.7, respectively), whereas removal of NO₃⁻ had relatively little effect (Δ AICc = 1.2). In remaining cases, covariate removal improved model quality (Δ AICc=-0.2 to -6.7), i.e., these covariates were not important in explaining variation in recruitment.

To depict the linkage between native perennial recruitment and litter abundance across all treatments, we combined litter cover and litter biomass measures into a principal component and treated this as covariate in a model that also included a quadratic term to account for the observed shift in pattern at low vs high litter abundance. This exercise showed a significant unimodal relationship wherein perennial



Figure 5. Relationships between recruitment of perennial seedlings in 2018 and litter abundance. Litter abundance was measured by **a** litter cover for treatments representing native or naturalized annuals and controls, **b** litter biomass for the invasive annual treatment, and **c** a principal component combining both litter metrics to visualize the unimodal relationship across all treatments (this relationship was also significant when we ran a parallel model using 2017 data: $F_{1,97} = 17.5$, P < 0.001). Photo shows a perennial forb (*Balsamorhiza sagittata*) seedling emerging amidst sparce litter in a naturalized plot. Note that recruitment was modeled with a negative binomial distribution in all cases and associated predicted relationships have been back-transformed to the original scale.

seedling recruitment increased at lower litter abundances represented by non-invasive annual and control treatments but decreased at higher litter abundances represented by the invasive annual treatment ($F_{1.97}$ = 21.4, P < 0.001; Fig. 5c).

Pathogen spillover experiment

For the dominant native perennial grass, *P. spicata*, emergence of marked seeds planted into field plots in fall 2018 was reduced by > 25% in invasive annual vs other treatments ($F_{3,61} = 10.0$, P < 0.001), with no differences among the latter (Suppl. material 1: fig. S3). Similarly, survival of these emerging seedlings from fall 2018 to spring 2019 was depressed by > 55% in invasive relative to remaining

treatments ($F_{3,61} = 8.2$, P < 0.001), again with no differences among the latter (Suppl. material 1: fig. S3). Examination of unemerged *P. spicata* seeds (n = 289) in the lab revealed that significantly more plots had evidence of attack by the pathogen *Pyrenophora* in the invasive annual treatment (20% of n = 20 invasive plots) relative to remaining treatments (0% of n = 40 plots; Fisher's exact test: P = 0.001, n = 60), but this pathogen accounted for the fate of only 1% of seeds examined overall. We found no evidence of attack by *Fusarium* pathogens.

PSF experiments

In the greenhouse experiment designed to evaluate PSFs of individual focal annuals, growth across all species in round 2 was 33% greater than in round 1 ($F_{1,68} = 94.1, P < 0.001$) while growth in inert soil did not differ between rounds overall ($F_{1,16} = 0.7, P = 0.41$), indicating that plants tended to generate soil conditions that favored subsequent growth. However, this effect on focal annual growth did not differ among native, naturalized, and invasive annuals (round x annual type: $F_{2,68} = 0.7, P = 0.41$; Suppl. material 1: fig. S4a). Similarly, growth of six native perennial species did not differ in soil trained with the invasive annual, *B. tectorum*, vs soil trained with native or naturalized annuals in our second greenhouse experiment (soil type: $F_{1,71} = 2.4, P = 0.13$; soil type x species: $F_{5,71} = 0.2, P = 0.94$; Suppl. material 1: fig. S4b).

Discussion

Explaining how some introduced plants become problematic pests is a central question in invasion ecology. Here, we explored this question by contrasting the roles that native, naturalized, and invasive annuals play in the context of local succession rules in perennial grasslands. We found that invasive annual bromes, including both cheatgrass and Japanese brome behaved quite differently from native and naturalized annuals. Native and naturalized annuals both developed ephemeral populations and facilitated establishment of the climax perennial natives, transitioning the community from early seral annuals to perennial dominance, consistent with succession theory. In contrast, the invasive bromes established robust populations that strongly inhibited recruitment of native perennials, thereby impeding succession. In essence, the invasive annuals acted as both early seral and climax species, thereby breaking local assembly "rules" for successional processes allowed us to identify traits like litter accumulation that may facilitate invasiveness but are not readily apparent using traditional trait comparisons.

In establishing the baseline for our system, we found that native annuals exhibited two functional behaviors that transitioned the community toward climax. First, they failed to maintain community dominance where sown, readily ceding space to other species (Fig. 1). Second, they facilitated establishment of perennial seedlings relative to unseeded control plots (Fig. 2). In sum, early seral natives actively transitioned the system toward climax in a Clementsian fashion (Clements 1916). Yet, naturalized annuals behaved in the very same manner, implying that the mechanisms underlying these patterns are generic in our system, more consistent with a Gleasonian perspective (Gleason 1926). This behavior of ceding space to other species is consistent with the idea that early seral species may experience stronger negative PSFs

(Kardol et al. 2006), which could provide a generic mechanism for this pattern, but we found no evidence for such PSFs in greenhouse experiments (see below). In sharp contrast, our invasive annual bromes maintained dominant populations at high biomass and suppressed establishment of native perennial seedlings, thereby impeding succession toward the native perennial state. This divergent behavior between invasive and naturalized annuals prevailed despite interannual variation in cover of sown annuals (Fig. 1) and recruitment of sown perennial seedlings (Fig. 2a) attributable to variation in weather conditions. While our experiment was relatively short, there was sufficient time for succession to progress to perennial dominance in the native and naturalized plots. Importantly, these findings are consistent with widespread accounts of annual bromes overtaking and dominating perennial grassland communities over vast expanses of the Intermountain West and Great Plains (Daubenmire 1942; Humphrey 1945; Mack 1981; Knapp 1996; Ogle et al. 2003; Germino et al. 2016; Pearson et al. 2016). Our results beg the question, how do these two groups of superficially similar introduced plants (all fast-growing, high-fecundity, annuals) generate such divergent outcomes in the recipient community?

In evaluating plausible explanations for these patterns, we examined covariates in the succession experiment and conducted additional experiments testing for pathogen spillover and PSFs (Fig. 6). Within our succession experiment, the invasive bromes generated higher live plant cover/biomass, litter cover/biomass, and available N (NH₄⁺ and NO₃⁻) and modestly reduced soil moisture relative to other treatments. A multivariate model including these covariates indicated that native perennial recruitment had strong negative relationships with litter abundance and NH₄⁺ in brome plots, with minimal evidence for live cover/biomass or soil moisture effects when accounting for these other factors. Interestingly, litter exhibited positive correlations with seedling recruitment at the low abundances generated by the native and naturalized annuals and negative correlations at the high abundances generated by the invasive annuals. The result was a unimodal relationship between litter abundance and native perennial seedling recruitment across treatments (Fig. 5). This pattern could be driven by physical properties of litter affecting



Invasive annuals inhibit grassland succession

Figure 6. Potential mechanisms allowing invasive annual bromes to inhibit grassland succession, as explored in our study. Summarized are 1) significant effects of invasive bromes (*Bromus japonicus* and *B. tectorum*) on biotic and abiotic factors, measured relative to treatments established with native and naturalized annuals, and 2) primary linkages between studied factors and recruitment of sown native perennials revealed through multivariable modeling and secondary experiments testing for pathogen spillover and PSFs (greenhouse only). Signs indicate direction of effects. Note that pathways are not necessarily independent. Collective effects of invasive annual bromes, including excessive litter buildup, prevented the transition to native climax perennials and maintained communities in a novel seral-climax state (see Discussion for details).

seedling germination and/or survival in ways that could be beneficial at low litter levels, explaining observed facilitative effects of native and naturalized annuals, but detrimental at higher levels (Carson and Peterson 1990; Meyer et al. 2014; see reviews in Facelli and Pickett 1991; Loydi et al. 2013). While litter abundance could influence soil moisture in ways that affect seedlings, our analysis of recruitment patterns indicated that litter abundance was important even when soil moisture was controlled for, with the latter explaining no significant variation in multivariate models (Suppl. material 1: table S5). Hence, it was unclear from the factors that we measured by what mechanism litter might influence native perennial seedlings. Studies documenting negative effects of Bromus litter on native recruitment in other systems have implicated light limitation (Meyer et al. 2014; Molinari and D'Antonio 2020), but thick litter may also form a mechanical barrier to seedling establishment (Facelli and Pickett 1991; Jessen et al. 2023). In contrast, litter manipulation studies show that establishment and performance of annual bromes are facilitated by litter buildup (Evans and Young 1970; Meyer et al. 2014; Molinari and D'Antonio 2020). Whatever the specific mechanism, high litter abundance appeared to favor brome dominance by inhibiting native perennial establishment while allowing bromes to recruit sufficiently to maintain robust populations.

Of course, the link between litter and perennial recruitment is potentially confounded with litter chemistry since the species producing the litter differed among treatments. In terms of litter quality, many introduced plants have higher leaf N (low C:N ratio) than co-occurring natives, which has been linked to increased litter decomposition rates and elevated soil N (Liao et al. 2008). However, B. tectorum litter has relatively low N (high C:N ratio; McLeod et al. 2021), a trait likely common to annual brome grasses and linked to slower decomposition, greater litter accumulation, and reduced soil N (Evans et al. 2001; Bansal et al. 2014). Although such a reduction in N inputs from litter could cause reduced seedling performance, we found that soil N was actually elevated in brome plots (see below), and effects of litter abundance on perennial recruitment were independent of soil N (Suppl. material 1: table S5). Alternatively, litter could suppress seedlings by leaching phytotoxins (Facelli and Pickett 1991), but field and greenhouse experiments evaluating the chemical effects of introduced annual Bromus litter on grassland species emergence have shown negligible effects (Amatangelo et al. 2008; Chen et al. 2018; Molinari and D'Antonio 2020). Collectively, these studies in combination with our results suggest that chemical properties of Bromus litter were unlikely to play a direct role in suppressing native plant recruitment, although they likely influenced litter buildup.

Numerous studies have linked annual bromes to elevated soil N (Paschke et al. 2000, Stark and Norton 2015; McLeod et al. 2016), but how they elevate N has been unclear. Recent work in our system indicates that *B. tectorum* is associated with a greater abundance of ammonia-oxidizing bacteria, which convert NH_4^+ to NO_3^- (McLeod et al. 2016). If annual bromes elevate N over long time periods, this can generate soil legacies that inhibit succession and impede restoration because elevated N favors annuals over perennials (see Paschke et al. 2000; Mazzola et al. 2011). However, the N levels we measured in brome plots were comparable to those observed in native grasslands in our system (McLeod et al. 2016), and hence would not be predicted to reduce seedling establishment. Yet NH_4^+ correlated negatively with native perennial recruitment in brome plots (evident even when we controlled for other measured factors). We found no plausible explanation for this pattern (NH_4^+ was not correlated with measured variables other than NO_3^-), but

we note that elevated N likely promoted *Bromus* populations (e.g., Paschke et al. 2000; Mazzola et al. 2011; Piper et al. 2015; Stark and Norton 2015) in a positive feedback process that fed the production of large quantities of litter (biomass of *Bromus* species and litter were positively correlated: r = 0.63, P = 0.003), which in turn suppressed perennial seedling recruitment.

Despite the potential for PSFs to strongly influence both succession and invasion processes, we found no evidence in our greenhouse experiments that PSFs differentially affected growth of Bromus relative to other annuals or that growth of native perennials was suppressed in soil trained by cheatgrass vs. other annuals. Whereas we might expect all the annuals to have negative feedbacks as predicted in the context of succession (Kardol et al. 2006; Kulmatiski et al. 2008) or the invasives alone to have strong positive feedbacks due to reduced enemies and/or more beneficial associations with mutualists (Klironomos 2002; Callaway et al. 2004; Levine et al. 2006), if anything, we found evidence of positive feedbacks that were comparable among native, naturalized, and invasive annuals (Suppl. material 1: fig. S4a). Likewise, while the highly invasive bromes could inhibit succession by reducing the abundance of arbuscular mycorrhizal fungi (Lekberg et al. 2013) that benefit late successional perennial species more than ruderal annuals (Wilson and Hartnett 1998), we did not find evidence for feedbacks on native perennials (Suppl. material 1: fig. S4b). Although our greenhouse results suggest that PSFs did not explain patterns seen in our field experiment, such lack of concordance could be attributed to complexities linked to PSF experiments (Brinkman et al. 2010; Forero et al. 2019).

Pathogen spillover from annual bromes by black fingers of death (*Pyrenophora semeniperda*) may periodically suppress native grass establishment in invaded stands (Beckstead et al. 2010, 2016; Meyer et al. 2014; but see Mordecai 2013). Although examination of marked seeds of *P. spicata*, the dominant native perennial grass in our system, revealed that attack by *Pyrenophora* was more likely in *Bromus* plots, providing evidence of pathogen spillover, this pathogen accounted for failed emergence of very few recovered seeds. Similarly, we did not find evidence of attack by other pathogens like *Fusarium* spp. Yet, the carefully monitored *P. spicata* seeds revealed that both seedling emergence and survival were strongly suppressed in brome plots. Moreover, most seeds that failed to emerge also showed no evidence of germination (93% of n = 289) upon inspection in the lab. These results suggest that factors such as excessive litter levels, which can influence both germination cues and seedling survival, were more important than seed pathogen spillover in explaining *Bromus* impacts on perennial recruitment in our experiment.

Cheatgrass is the most notorious invasive plant in the western United States (Mack 1981; Knapp 1996; Bradley et al. 2018). Hence, understanding how this species and other annual bromes achieve community dominance is critical to mitigating their impacts. Cheatgrass has been linked to "grass-fire" cycles in the western United States wherein it generates abundant fine fuels that increase fire frequency, thereby favoring cheatgrass over natives, which are poorly adapted to frequent disturbance (D'Antonio and Vitousek 1992; Balch et al. 2013; Bradley et al. 2018). Such fire feedbacks could explain cheatgrass dominance if fire return intervals increased sufficiently to lock the system into an early seral state, such as the 3–5 year fire return intervals described for 9 sites in Idaho by Whisenant (1990). However, modeling approaches applied over vast regions of the Intermountain West have reported fire return intervals of 50–78 years in cheatgrass-invaded sites (Balch et al. 2013). Although these intervals translate to a marked increase in fire frequencies,

the period between fires should be more than sufficient for intermountain grasslands to reach climax. In our plots, perennial dominance over native and naturalized annuals was achieved in a few growing seasons. While cheatgrass's influence over fire regimes undoubtedly favors its expansion over large landscapes, this process is insufficient to explain how this annual achieves dominance over perennial grasslands. We hypothesize that cheatgrass overtakes native communities by way of a "ratcheting" effect wherein it invades recent disturbances and then dominates newly claimed ground by inhibiting succession. One study sowing cheatgrass into experimentally disturbed vs. control plots demonstrates this species' initial reliance on disturbance, showing that while cheatgrass thrives in disturbed plots, it performs very poorly in undisturbed plots (Pearson et al. 2023). Our hypothesis is consistent with historical accounts describing the progression of cheatgrass invasion as a ratcheting effect wherein disturbances like grazing and human activities facilitate initial invasions that then shift systems from perennial to annual dominance (note that early accounts do not invoke fire, see Mack 1981 and citations therein). We propose that cheatgrass overtakes perennial intermountain grasslands, shifting them to a novel alternative equilibrium state (sensu Hobbs et al. 2006) that we refer to as a seral-climax invasive community, by impeding succession following disturbance via processes linked to litter buildup.

Traditional approaches to understanding how some introduced species become problematic pests have compared traits among invasive, naturalized, and native species without reference to the local assembly rules that define recipient communities. Such studies have broadly linked invasive species to traits like high fecundity and rapid growth (Pysek and Richardson 2007; Van Kleunen et al. 2010; Jelbert et al. 2015; Moracová et al. 2015), but they do not explain why many introduced plants bearing similar traits are benign. While the introduced plants in our study exhibit superficially similar traits in that they are all ruderal annuals with rapid growth and high fecundity, the invasive and naturalized taxa demonstrated highly divergent behaviors when evaluated in the context of local assembly rules. The naturalized annuals closely followed local assembly rules to behave like the natives, explaining why these species do not overtake recipient communities. In contrast, the invasive annuals broke local assembly rules by impeding succession to act as both early seral and climax, or "seral-climax," species. The invasive bromes appeared to inhibit succession by producing large quantities of litter that suppressed native perennial seedling establishment. While we were unable to identify the specific mechanisms underlying these inhibitory effects, they likely involve some aspect of ecosystem engineering. Notably, many of the worst invaders (including B. tectorum) have been linked to ecosystem engineering effects wherein the invader alters availability of resources like nutrients, moisture, light, etc. for other species (Crooks 2002). While obviously important, engineering effects may not always be amenable to ex situ comparisons typically applied to classic traits like seed mass, plant height, specific leaf area, etc. because the relevance and strength of engineering effects are explicitly measured at the local community scale. More generally, litter production may be considered an extended phenotype (sensu Dawkins 2016) much like spider webs and other engineering traits that are not conducive to traditional ex situ trait comparisons because they manifest as a function of environmental context. Contrasting native, naturalized, and invasive plants in the context of local assembly processes (sensu Pearson et al. 2018a) provides a powerful means for elucidating invader advantages, including mechanisms not readily amenable to traditional ex situ trait comparisons.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

DEP and YKO initiated the project and developed and implemented the field experiment. YL led in developing the plant-soil-feedback experiments, soil nutrient sampling, and evaluation of seed pathogens. YKO analyzed the data. All authors contributed to the writing.

Data availability

Data is available in Dryad: https://doi.org/10.5061/dryad.9ghx3fft6.

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Supplementary material 1

Additional results and study design information for field and greenhouse experiments

Authors: Dean E. Pearson, Yvette K. Ortega, Ylva Lekberg

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Data Paper

More than half of the alien plants naturalised in the arid southeast of the Iberian Peninsula could be invasive

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Abstract

Having a list of alien plant species naturalised in an area and knowing their invasive potential (i.e. a post-border species risk assessment framework) and the precise locations where they are found, are now a priority as a management strategy to curb their spread, avoiding damage to ecosystems and saving management costs. This is especially important in arid ecosystems, which are particularly vulnerable to impacts due to their limited resources. Weed Risk Assessment systems (WRAs) analyse plant traits that influence their invasive potential through a set of questions whose answers score taxa according to their invasive potential. In this work, we identify potentially invasive plants inhabiting the arid southeast of the Iberian Peninsula, the driest region in Europe, by compiling alien plant species recorded in the wild and applying the Australian and New Zealand Weed Risk Assessment (AWRA) system. The AWRA applies scores that evaluate species characteristics related to biography, undesirable attributes and biology/ecology for establishment elsewhere. We provide the dataset obtained in the application of the AWRA test: a list of the alien plant species naturalised in the study area and their geographical distribution; the answers, scores and results of the test, as well as the scientific sources that support the existence of such characteristics in these species. We found that 64.4% of the 177 taxa assessed can be considered potential invaders. This database represents a useful and transparent tool for environmental managers to deal with the problem of plant invasions effectively. It can also be confronted with data from other areas of the world where these species are naturalised.

Key words: Mediterranean dryland, plant invasive species, post-border analyses, Weed Risk Assessment (WRA)

Introduction

Plant invasions compromise all types of ecosystem services through changes in components, structure and functions of the ecosystems (Charles and Dukes 2008). Apart from the loss of nature contributions to humans, these alterations usually lead to great economic costs for governments (e.g. Haubrock et al. (2021a, b)), with management costs an important part of them (e.g. Angulo et al. (2021)). Several studies point out that arid ecosystems are more resistant to plant invasion, as native plant species seem to be better adapted to their critical conditions: resources-limited environments with a variable and unpredictable precipitation



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regime (Drake 1988; Loope et al. 1988; Chytrý et al. 2008). Despite this, invasive plant species have largely increased in arid regions over the last decades, even in those areas where management and monitoring strategies are in place (Shackleton et al. 2020). Some alien plants have the potential for overcoming ecosystem-poor conditions (Ozaslan et al. 2016) and the ability to use limiting resources more effectively than native plants during critical periods of the life cycle (Funk and Vitousek 2007; González-Rodríguez et al. 2010; Salinas-Bonillo et al. 2023). This behaviour would be exacerbated under future climate change scenarios (Ali and Bucher 2022). In addition, the highly productive and resource-rich areas of arid regions (e.g. river basins) are often overexploited by human activities, making them particularly sensitive to plant invasions (Milton and Dean 2010). Therefore, plant invasions occur in arid areas, causing severe disturbances in very vulnerable ecosystems, which are often simultaneously subject to other drivers of global change (Tylianakis et al. 2008; D'Odorico et al. 2013). Consequently, there is a need to identify potential plant invaders to prevent their introduction (i.e. pre-border weed risk assessment framework) and make more efficient management decisions (Seebens et al. 2017). In addition, once alien plants have already been introduced into the wild (i.e. post-border species risk assessment framework), managers need tools to prioritise management actions to eradicate, prevent the spread and control those most damaging to ecosystems (Early et al. 2016; McGeoch et al. 2016).

Weed Risk Assessment (WRAs) systems have proven to be a cost-effective and successfully tested pre-border tool for predicting the invasiveness of alien plants in various parts of the world (Tucker and Richardson 1995; Reichard and Hamilton 1997; Pheloung et al. 1999; Weber and Gut 2004; Parker et al. 2007; Gassó et al. 2010). Such assessments are also very useful for setting management priorities of plant species in a post-border scenario (Randall et al. 2008; Crosti et al. 2010; Gassó et al. 2010). In particular, the Australian and New Zealand Weed Risk Assessment (AWRA) system (Pheloung et al. 1999) has shown high applicability and predictive power in many regions (Gordon et al. 2008; Nishida et al. 2009; Crosti et al. 2010; Gassó et al. 2010; McClay et al. 2010; Koop et al. 2012), including arid areas. Despite WRAs having been questioned for being time-consuming and eventually lacking predictive value for some species (Hulme 2012; Kumschick and Richardson 2013), they have been useful for rejecting invasive species (Gassó et al. 2010), showing they are a suitable tool for managers to obtain blacklists, for instance. In this work, we aimed to identify and classify potential plant invaders amongst the naturalised alien plant species in the arid south-eastern region of the Iberian Peninsula, the driest region in Europe (Alcaraz 2017), applying the AWRA test. Our specific objectives were: i) to define a list of naturalised alien taxa in the study area, ii) to identify and classify species according to their invasive potential, iii) to document traits that contribute to their invasiveness and iv) to provide their known geographical coordinates to contribute to global and local information on hotspots expansion. To ensure transparency and allow for verification of the information obtained, we provide the references consulted to answer the test questions. We believe that this database of naturalised alien plant species in an arid zone is a useful tool for researchers on biological invasions and also for managers engaged in the monitoring and management of this environmental problem in these areas.

Metadata

I. Dataset descriptors

A. Dataset identity

Scores of the Australian and New Zealand Weed Risk Assessment (AWRA) (Pheloung et al. 1999) for 144 alien plant species naturalised in the arid southeast of the Iberian Peninsula, the geographical coordinates where they are recorded in the wild and the bibliographic sources from which the information was obtained to answer the AWRA questions.

B. Dataset identification code

AWRAridSpain_*.csv.

C. Dataset description

The dataset consists of five semicolon-separated values (.csv) files (Table 1).

Table 1. Description of the five files that include the dataset AWRAridSpain_*.csv.

File name	# Rows (excluding the header)	# Columns
AWRAridSpain_dic_taxa.csv	177	8
AWRAridSpain_dic_questions.csv	49	4
AWRAridSpain_dic_references.csv	217	2
AWRAridSpain_answers	8,673	5
AWRAridSpain_species_location	512	6

1. Principal investigators

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II. Research origin descriptors

A. Overall project description

1. Identity

We compiled data on the alien plant species naturalised in the arid southeast of the Iberian Peninsula and conducted the Australian and New Zealand Weed Risk Assessment (AWRA) (Pheloung et al. 1999) for each one of them. We also provide data on their botanical family and their time of entry (archaeophytes or neophytes *sensu* Richardson et al. (2000)), the known geographical coordinates where they are recorded in the wild and the bibliographic sources from which the information was obtained to answer the AWRA questions.

2. Originators

Javier Cabello and María J. Salinas-Bonillo conceived the idea, Miguel Cueto led the development of the plant species distribution database, Javier Cabello, Alba Rodríguez-Rodríguez, María J. Salinas-Bonillo and M. Trinidad Torres-García carried out the analyses. Alba Rodríguez-Rodríguez and María J. Salinas-Bonillo reviewed and organised all the databases. Javier Cabello obtained funding. All authors contributed to the writing of the manuscript.

3. Period of study

Data collection and analysis were conducted over the duration of the two projects within which this work was carried out (2021–2023) (See Sources of funding section).

4. Objectives

We mainly aimed to identify and classify potential plant invaders amongst the naturalised alien plant species in the arid south-eastern of the Iberian Peninsula by applying the AWRA test. The specific objectives were: i) to list naturalised alien taxa in the study area, ii) to identify and classify species according to their invasive potential, iii) to document traits that contributing to their invasiveness and iv) to provide their known geographical coordinates to contribute to global and local information on hotspots expansion. To ensure transparency and allow verification of the information obtained, we provide the references consulted to answer the test questions. This database of naturalised alien plant species in an arid zone is a useful tool for researchers on biological invasions and for managers engaged in monitoring and managing this environmental problem in these areas.

5. Sources of funding

This work has been performed within the projects "Scientific infrastructures for global change monitoring and adaptation in Andalusia (LIFEWATCH-INDALO)" (LIFEWATCH-2019-04-AMA-01) and "Indicators for monitoring the supply and demand of ecosystem functions and services of the Complementary Research & Development & Innovation Plan of the Biodiversity area (SP4-LiA3)", both funded by the European Union. This research was also done within the LTSER platform "The Arid Iberian South East LTSER Platform," Spain (LTER_EU_ES_027).

B. Specific subproject description

1. Site description

The area of study comprises the arid regions of Andalusia, southeast of Spain (36°46'N, 1°40'W to 37°29'N, 3°07'W; 1,220.7 ha, Fig. 1), delimited according



Figure 1. Map of the study area (arid regions of Andalusia, southeast of the Iberian Peninsula) showing the density of alien plant species records.

to the ecoregionalisation map of the Network of Protected Natural Spaces of Andalusia (Montes et al. 1998; Requena-Mullor et al. 2018). The altitudinal gradient ranges from 0 to 2,040 m a.s.l. The predominant climate is warm and dry Mediterranean, with average annual temperatures between 12 and 18 °C and annual rainfall between 200 and 350 mm, although in some areas it can be lower (Armas et al. 2011). The geology is diverse, with many rock types such as gypsum, limestone, marl, phyllite, quartzite, schist and volcanic rocks (Armas et al. 2011; Alcaraz 2017). The climate favours soils with high CaCO₃ concentrations, low organic matter and nutrient contents, reduced aggregate stability and low water retention capacity (Armas et al. 2011; Alcaraz 2017). The most frequent vegetation types are high scrublands and scattered low scrublands and perennial grasslands with Macrochloa tenacissima (L.) Kunth as a common species (Cabello et al. 2012; Alcaraz 2017). In addition, the mountainous areas in arid Andalusia host evergreen forests of Quercus spp. and reforestations of Pinus spp. There is a contrasting riparian vegetation, from the source of the watercourses (mainly deciduous trees) to their mouths (with evergreen shrubs and tall halophytes as dominant species) (Salinas et al. 2000a, b; Salinas and Casas 2007; Alcaraz 2017). Despite its arid nature, this region harbours high levels of biodiversity, with numerous endemic species and habitats of conservation concern at European levels (Armas et al. 2011; Sánchez-Piñero et al. 2011; Mendoza-Fernández et al. 2014). Most of the economic activities are related to greenhouse horticulture and its parallel industries such as packaging and transport, seed and seedling production or biological control and the tourism and service sectors (Sánchez-Picón et al. 2011; Piquer-Rodríguez et al. 2012;

Requena-Mullor et al. 2018). In particular, the intensification and mechanisation of agriculture contributed greatly to the increase in population (Aznar-Sánchez et al. 2011; Quintas-Soriano et al. 2016), which, together with urban development, especially in coastal zones, made this area one of the most transformed in Spain (Quintas-Soriano et al. 2016). Parallel to these extensive land transformations of the territory, a simultaneous effort has been made to protect natural areas with remarkable biodiversity, with more than 30 protected areas having been declared in the last decades, with the current percentage of conserved land area standing at 20% (Quintas-Soriano et al. 2016).

2. Research methods

AWRA test

We created the list of alien species naturalised in the study area using the most updated plant database for eastern Andalusia compiled in the Florandor project (Blanca et al. 2009). Florandor involved intensive field plant collection, institutional herbarium data collection and species identification in which botanists from four Spanish universities in south-eastern Andalusia worked. Then we implemented the AWRA test (Pheloung et al. 1999) to all taxa in the list, consisting of 49 questions divided into three sections related to biography, undesirable attributes and biology/ecology. To answer the questions, we followed the Gordon et al. (2010) guidelines and used references about regional flora and invaders and several online resources (see AWRAridSpain_dic_references.csv). We classified each taxon according to its AWRA score. The scoring system classifies the analysed taxa in three groups according to recommendations for the entrance of the alien plant to the country (Pheloung et al. 1999): "reject" for taxa with a score higher than 6, "accept" for taxa with scores lower than 1 and "evaluate" for taxa with a score between 1 and 6, as they would require further evaluation. According to our objective, we considered the "reject" taxa as the "potential invaders". Given that the time elapsed since the arrival of a species in a territory (i.e. residence time status, Pyšek et al. (2012)) influences the expansion and invasive behaviour (Pyšek et al. 2004, 2005), we differentiated allochthonous plants introduced by humans in prehistoric times (i.e. archaeophytes) from those that arrived recently (i.e. neophytes). In Europe, these terms refer to taxa introduced before or after 1492, respectively (Richardson et al. 2000).

Despite the fact that we could have answered the minimum questions required by Pheloung et al. (1999) for each species, we tried to answer as many as possible if enough information were available (Table 2). However, when we had insufficient information, we introduced "NA" (not answered, see section IV, A, 6. Special characters/fields). We answered a minimum of 20 questions for each taxon and a maximum of 42, with 33.2 ± 6.0 responses on average (± standard deviation). For all taxa, we answered more than the minimum number of questions required for each section: 4 from sections A (Biogeography) and B (Undesirable attributes) and 8 from section C (Biology/ecology). Regarding the topic, the mean number of questions answered per taxon was: 5.4 ± 1.5 (out of 8), 7.6 ± 1.8 (out of 10) and 17.7 ± 3.3 (out of 28) for the Agricultural, Environmental and Combined questions, respectively.

	Section				Total		
	Α	В	С	Agricultural	Environmental	Combined	10141
Minimum	4	4	8	2	3	10	20
Maximum	13	12	21	8	10	24	42
Mean ± SD	9.4 ± 2.2	8.3 ± 1.8	15.4 ± 3.1	5.4 ± 1.5	7.6 ± 1.8	17.7 ± 3.3	33.2 ± 6.0

Table 2. The minimum, maximum and mean number of questions answered for each taxon according to section, topic and total. SD: Standard deviation.

For questions 2.01 and 2.02, we scored "2", as Gordon et al. (2010) recommended when no climate analysis is performed. This explains why we did not add a reference for these questions ("NR", not reference, see section IV, A, 6. Special characters/fields).

We added "not evidenced" in the reference field for questions answered with "no" when there is no evidence for the affirmative ("yes") answer in the literature, for example, Question 5.01 (Aquatic) for terrestrial species and that fact is not specified in the literature.

We registered and evaluated 177 taxa of alien naturalised species in the study area. Some 64.4% of the taxa could be considered potential invaders, 9.6% could be regarded as harmless taxa, and 26.0% would need further evaluation (Table 3).

Table 3. Total number and percentage of the outcomes obtained for the 177 taxa analysed.

Outcome	Accept (<1)	Evaluate (1–6)	Reject (>6)	
Total number	17	46	114	
%	9.6	26.0	64.4	

Spatial distribution of alien species

We used the geographic coordinate data from the Florandor project (Blanca et al. 2009) to construct a geographical coordinate database and a density map of the records of alien species in the study area (Fig. 1). We made this map from the vector layer of record points using the "Density Analysis" plugin (ID 2717) in QGIS 3.22.7. We used a cell size of 10×10 km and divided the cells into six classes of equal intervals (except for the zero class).

Project personnel

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III. Data-set status and accessibility

A. Status

1. Latest update

02/04/2024.

2. Metadata status

The metadata were last revised and updated on 2 April 2024.

3. Data verification

We checked exhaustively the data before publication. The plant species names were cross-checked with the Blanca et al. (2009) flora guide. We have also corrected some of coordinates and descriptions of the species locations and converted UTM coordinates to geographic coordinates.

B. Accessibility

1. Storage location and medium

The data-set is available on the Zenodo repository (DOI: 10.5281/zenodo.10790372) under a Creative Commons Attribution 4.0 International Licence (CC-BY 4.0).

2. Contact persons

María J. Salinas-Bonillo: mjsalina@ual.es Javier Cabello: jcabello@ual.es

3. Copyright restrictions

This data-set can be freely used for non-commercial purposes.

4. Proprietary restrictions

This data-set is licensed under a Creative Commons Attribution 4.0 International Licence (CC-BY 4.0). We request that users of these data cite this data paper in any publications resulting from its use. The authors are available for consultations about and collaborations involving the data.

IV. Data structural descriptors

A. Data-set file

1. Identity

Since we provided data from different entities with the application of the AWRA test, we structured the dataset in a relational database consisting of five linked tables (Fig. 2).

- a. AWRAridSpain_dic_taxa
- b. AWRAridSpain_dic_questions
- c. AWRAridSpain_dic_references
- d. AWRAridSpain_answers
- e. AWRAridSpain_species_location

2. Size

- a. AWRAridSpain_dic_taxa: 177 rows (excluding the header), 8 columns, 15.9 kbytes.
- b. AWRAridSpain_dic_questions: 49 rows (excluding the header), 4 columns, 2.3 kbytes.
- c. AWRAridSpain_dic_references: 217 rows (excluding the header), 2 columns, 48.4 kbytes.
- d. AWRAridSpain_answers: 8,673 rows (excluding the header), 5 columns, 233.9 kbytes.
- e. AWRAridSpain_species_location: 512 rows (excluding the header), 6 columns, 69.34 kbytes.



Figure 2. Scheme showing the structure of the AWRAridSpain database model. PK and FK stand for primary key and foreign key, respectively. PK is the unique identifier of each table and the FK refers to the primary key of a different table, which links the two tables.

3. Format and storage mode

The five tables are available as semicolon-separated values (.csv) files and the vector layer including the geographical location of the species in the study area (see Related materials below) as shapefile format (.shp). All the files are compressed as a one zip Archive (.zip). We created the semicolon-separated values (.csv) files with UTF-8 code as follows:

- 1. First, we saved our excel (.xlsx) files as unicode plain text (.txt) files.
- 2. Then, we replaced "tabs" with "semicolon".
- 3. Finally, we saved the unicode plain text (.txt) files as semicolon-separated values (.csv) with UTF-8 code.

4. Header information

- a. AWRAridSpain_dic_taxa: See Table 4.
- b. AWRAridSpain_dic_questions: See Table 5.
- c. AWRAridSpain_dic_references: See Table 6.
- d. AWRAridSpain_answers: See Table 7.
- e. AWRAridSpain_species_location: See Table 8.

5. Special characters/fields

"NA (not answered)" indicates that the question was not answered in the AW-RAridSpain_answers table.

"NR (no reference)" indicates that the question does not need a source, in the AWRAridSpain_answers table.

"NT (no questionType)" indicates that the question does not belong to any type, in the AWRAridSpain_dic_questions table.

B. Variable information

Table 4. Header information of "AWRAridSpain_dic_taxa.csv".

Definition	Values range (minimum, maximum)
Unique identifier of the taxon	1–177
Taxon name with author names	_
Parts of the taxon name separated by an underscore and without authors' names	_
Authorship information for the taxon name	_
Scientific name of the family in which the taxon is classified	_
Neophyte vs. Archaeophyte	_
Score obtained for the taxon in the AWRA test	-6-31
Invasive potential of the taxon based on the recommendation given by the	-
	Definition Unique identifier of the taxon Taxon name with author names Parts of the taxon name separated by an underscore and without authors' names Authorship information for the taxon name Scientific name of the family in which the taxon is classified Neophyte vs. Archaeophyte Score obtained for the taxon in the AWRA test Invasive potential of the taxon based on the recommendation given by the score: Beject vs. Evaluate vs. Accent (see the Besearch Methods section)

 Table 5. Header information of "AWRAridSpain_dic_questions.csv".

Field name	Definition	Values range (minimum, maximum)
questionID	Unique identifier of the question. We used the same number as in Pheloung et al. (1999)	1.01-8.05
question	The full question as in Pheloung et al. (1999)	_
questionType	Question type according to Pheloung et al. (1999): A (Agricultural) vs. E (Environmental) vs. C (Combined). NT = no questionType	-
section	Section to which question belongs according to Pheloung et al. (1999): A (Biogeography) vs. B (Undesirable attributes) vs. C (Biology/ecology)	-

Table 6. Header information of "AWRAridSpain_dic_references.csv".

Field name	Definition
referenceID	Unique identifier of the reference consisting of the short name of the resource (paper or website) where the answer to the question was found.
fullReference	Full name of the resource (paper or website) where the answer to the question was found.

Table 7	TT 1	· c ·	C	"ATV/D A . 10	•	"	
Table 7.	Header	information	of	AWKArids	pain	answers.csv.	
			~ ~		P		

Field name	Definition	Values range (minimum, maximum)
taxonID	Unique identifier of the taxon	1–177
questionID	Unique identifier of the question. We used the same number as in Pheloung et al. (1999)	1.01-8.05
answer	The answer given to the question: N (No) vs. Y (Yes). NA = Not answered.	_
answerScore	Score given to each answer. NA = Not answered.	-3-4
referenceID	Unique identifier of the reference consisting of the bibliographic source consulted to provide the answer. NA = Not answered.	_

Table 8. Header information of "AWRAridSpain_species_location.csv".

Field name	Definition	Values range (minimum, maximum)
locationID	Unique identifier of the species location point	1–512
taxonID	Unique identifier of the taxon	1–177
province	Province of the location point	_
location	Description of the location point place	_
longitude	Longitude of the species location point (EPSG:4326 - WGS 84) in degrees, minutes and seconds (DMS)	_
latitude	Latitude of the species location point (EPSG:4326 - WGS 84) in degrees, minutes and seconds (DMS)	_

V. Supplemental descriptors

A. Data acquisition

1. Data forms or acquisition methods

All the fields were taken directly on Excel sheets.

2. Data entry verification procedures

We revised our list of taxa so that the names matched those in the Florandor project (Blanca et al. 2009). We also revised the coordinates of the species location.

B. Related materials

We accompanied the dataset with a vector layer containing the georeferenced location points of the alien plant species, in shapefile format (.shp).

C. Computer programmes and data-processing algorithms

We used the Free and Open Source Software QGIS 3.22.7 to create the vector layer of the alien species record points, obtain the geographic coordinates in degrees, minutes and seconds and create the map in Fig. 1.

We employed the online app MIRO (https://miro.com/) to create the database model schema in Fig. 2.

D. Archiving

1. Archival procedures

The data-set will be permanently archived in the ZENODO repository specified above.

E. Publications and results

These data have helped the team to prioritise some studies on harmful invasive plant species in the southeast Iberian Peninsula.

Rubio-Ríos J, Pérez J, Fenoy E, Salinas-Bonillo, MJ Casas, JJ (2023) Cross-species coprophagy in small stream detritivores counteracts low-quality litter: native versus invasive plant litter. Aquatic Sciences 85: 8. https://doi.org/10.1007/ s00027-022-00905-z

Salinas-Bonillo MJ, López-Escoriza A, Cabello-Piñar J (2012) Expansion of the invasive plant species *Pennisetum setaceum* (Forssk.) Chiov in arid and semi-arid areas of eastern Andalusia (province of Almería). Technical report of the Programme for monitoring the effects of global change in arid and semi-arid areas of eastern Andalusia (GLOCHARID) 852/09/M/00 (2012). Natural Heritage, Biodiversity and Global Change Foundation, Almeria, Spain.

Salinas-Bonillo MJ, Torres-García MT, Paniagua MM, Sánchez MM, Cabello J (2023) Clonal mechanisms that matter in *Agave fourcroydes* and *A. sisalana* invasions in drylands: implications for their management. Management of Biological Invasions 14(1): 80–97. https://doi.org/10.3391/mbi.2023.14.1

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

This work has been performed within the projects "Scientific infrastructures for global change monitoring and adaptation in Andalusia (LIFEWATCH-INDALO)" (LIFEWATCH-2019-04-AMA-01), and "Indicators for monitoring the supply and demand of ecosystem functions and services of the Complementary Research & Development & Innovation Plan of the Biodiversity area (SP4-LiA3)", both funded by the European Union.

Author contributions

Javier Cabello and María J. Salinas-Bonillo conceived the idea, Miguel Cueto led the development of the plant species distribution database, Javier Cabello, Alba Rodríguez-Rodríguez, María J. Salinas-Bonillo, and M. Trinidad Torres-García carried out the analyses. Alba Rodríguez-Rodríguez and María J. Salinas-Bonillo reviewed and organised all the databases. Javier Cabello obtained funding. All authors contributed to the writing of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

AWRAridSpain_dic_taxa

Authors: María J. Salinas-Bonillo, Alba Rodríguez-Rodríguez, M. Trinidad Torres-García, Miguel Cueto, Javier Cabello

Data type: csv

- Explanation note: Semicolon-separated values (CSV) text file containing the record of the 177 species of alien plants naturalized in the study area indicating: scientific name, authorship, family and time of entry (archaeophytes or neophytes sensu Richardson et al. 2000, see main text file for complete bibliographic reference), the score obtained by each taxon in the AWRA test and the invasive potential of each taxon according to the recommendation given by the score: Reject vs Evaluate vs Accept (see Research Methods section in the main text file).
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- Link: https://doi.org/10.3897/neobiota.96.136154.suppl1

Supplementary material 2

AWRAridSpain_dic_questions

Authors: María J. Salinas-Bonillo, Alba Rodríguez-Rodríguez, M. Trinidad Torres-García, Miguel Cueto, Javier Cabello

Data type: csv

- Explanation note: Semicolon-separated values (CSV) text file containing the AWRA test questions indicating: the complete question according to Pheloung et al. (1999), see main text file for full bibliographic reference), the type of question (A, Agricultural, E, Environmental, C, Combined) or NT (no type) and the Section to which the question belongs according to Pheloung et al. (1999): (A, Biogeography, B, Undesirable attributes, C, Biology/ecology).
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Link: https://doi.org/10.3897/neobiota.96.136154.suppl2

Supplementary material 3

AWRAridSpain_dic_references

Authors: María J. Salinas-Bonillo, Alba Rodríguez-Rodríguez, M. Trinidad Torres-García, Miguel Cueto, Javier Cabello

Data type: csv

- Explanation note: Semicolon-separated values (CSV) text file containing the resources (articles or websites) in which each answer to the question has been found for each species, indicating a unique reference identifier consisting of an abbreviated name, in addition to the full reference.
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Link: https://doi.org/10.3897/neobiota.96.136154.suppl3

Supplementary material 4

AWRAridSpain_answers

Authors: María J. Salinas-Bonillo, Alba Rodríguez-Rodríguez, M. Trinidad Torres-García, Miguel Cueto, Javier Cabello

Data type: csv

- Explanation note: Semicolon-separated values (CSV) text file containing the scores of the questions answered by each species and the bibliographic sources consulted to provide the answer.
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- Link: https://doi.org/10.3897/neobiota.96.136154.suppl4

Supplementary material 5

AWRAridSpain_species_location

Authors: María J. Salinas-Bonillo, Alba Rodríguez-Rodríguez, M. Trinidad Torres-García, Miguel Cueto, Javier Cabello

Data type: csv

- Explanation note: Semicolon-separated values (CSV) text file containing the geographic location where the alien plant species have been recorded in the study area, indicating the province, the location point and the geographic coordinates (longitude and latitude in the spatial reference system EPSG:4326-WGS 84) in degrees, minutes and seconds.
- Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/neobiota.96.136154.suppl5

Supplementary material 6

Shapefile location

Authors: María J. Salinas-Bonillo, Alba Rodríguez-Rodríguez, M. Trinidad Torres-García, Miguel Cueto, Javier Cabello

Data type: zip

- Explanation note: Dataset with a vector layer containing the georeferenced location points of the alien plant species, in shapefile format (.shp).
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Contrasts in perception of the interaction between non-native species and climate change

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Abstract

Over the last century, intensification of human movement has resulted in a large-scale redistribution of species worldwide. In recent decades, this phenomenon has been further compounded by climate change, creating complex challenges in the management of non-native species. As effective management can be hampered by gaps in communication and understanding between scientific researchers, natural resource managers and the wider public, assessing consensus between these groups is crucial.

Here, we adopt an explorative approach to analyse three key groups concerned with the management of freshwater ecosystems – recreational fishers, natural resource managers and scientific researchers. Our objective is to better understand the level of consensus regarding the interaction between non-native species and climate change.

We found that, while scientific researchers and managers had varying opinions on the management of non-native species as driven by climate change, recreational fishers were almost unanimously opposed to the potential presence of non-native species, regardless of the nature of their introduction. Additionally, definitions of what constitutes a non-native species varied greatly between and within the groups.

Our results underline both the current lack of consensus on the definition and management of non-native species and gaps in understanding between and within the three groups regarding both the nature of non-native species and the range-shifting effects of climate change.

Key words: Climate change, non-native species, public perception

Introduction

Over the last century, intensification of human movement worldwide has resulted in a large-scale redistribution of species, a trend that is predicted to continue at a similar pace in the coming decades (Seebens et al. 2020). Most of the redistribution of these non-native species has historically been driven by human translocation (Mack et al. 2000; Carpio et al. 2019). If these non-native species become established and begin to spread, their local impacts can include population declines and even local extirpations of native species and restructuring of food webs (Mack et al. 2000; Gallardo et al. 2016). Human activity has also resulted in climate change, which can reduce species' populations through warming temperatures and an increase in



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the frequency of extreme weather events (IPCC 2021). In recent decades, climate change has compounded the effects of this redistribution of species. This can happen in a number of ways (Rolls et al. 2017). Firstly, through the direct movement – often termed 'range-shifting' – of species to higher latitudes and altitudes as a response to warming temperatures (Chen et al. 2011). Secondly, through allowing species to establish and spread upon introduction to ecosystems that were previously too cold for either process (Comte and Grenouillet 2013). Thirdly, through changing interactions between already-established non-native species and native species they had previously co-occurred with, such that native species are more negatively impacted (Gilman et al. 2010; Hein et al. 2014; Perrin et al. 2020a).

The complexity of the interaction between the non-native species and climate change leads to increasingly difficult management challenges. Successful management approaches, such as preventing the introduction of non-native species and conserving native species and communities, are dependent on three key groups – a) scientific researchers, who provide the research upon which management decisions are based (Pecl et al. 2017; Beaury et al. 2020), b) natural resource managers, who make and implement management decisions (Pietrzyk-Kaszyńska and Grodzińska-Jurczak 2015) and c) members of the public whose lives are affected by management decisions (henceforth referred to as 'the wider public'), whose approval is often necessary for the success of management decisions (García-Llorente et al. 2008; Gozlan et al. 2013; Verbrugge et al. 2013; Courchamp et al. 2017; Novoa et al. 2018; Deak et al. 2019; Kapitza et al. 2019; Kochalski et al. 2019).

Within research communities, there is substantial debate over both the terminology and management of non-native species, with traditionally popular terms such as 'invasive' and 'alien' viewed by some researchers as, at best subjective and, at worst pejorative (Shackelford et al. 2013; Head 2017; Abbate and Fischer 2019). The indirect role of climate change in range shifts in species means that range-shifting species are generally not termed as invasive or alien within the research community – though this is not always the case (Peterson and Robins 2003) – and there has been objection to the application of invasive frameworks to range-shifting species (Urban 2020). Here, we use the term 'non-native species' when referring to a term that is outside of its historically-defined native range.

Many natural resource managers (henceforth referred simply to as 'managers') have begun to incorporate the effects of climate change into management actions regarding non-native species (Rahel et al. 2008; Beaury et al. 2020), including habitat manipulation and restriction of dispersal in areas where native species are of conservation concern (Scheffers and Pecl 2019). However the strict maintenance of species assemblages in areas where the climate has rendered habitats unsuitable for native species may become resource-intensive and ultimately untenable (Scheffers and Pecl 2019). These difficulties are compounded by the fact that, amongst the wider public, climate change itself is enough of a controversial issue, with the phenomena sometimes rejected as a threat by members of the public, even those whose livelihoods are directly affected (van Baal et al. 2023). Knowledge of risks and management techniques amongst the wider public regarding non-native species also varies from region to region and over time (Bremner and Park 2007; Verbrugge et al. 2013; Ridbäck and Dietze-Schirdewahn 2017; Deak et al. 2019; IPBES 2023). Knowledge of a species' invasiveness and exposure to its negative impacts can increase negative perceptions of non-native species (Lindemann-Matthies 2016; Luna et al. 2019), yet these perceptions can often diminish over time as familiarity with them increases (Henke et al. 2024).

Gaps in communication or understanding between managers, scientific researchers and public stakeholders often hinder both the development and implementation of effective management policies (IPBES 2023; Gonzalez-Sargas et al. 2024; Kinsley et al. 2024), with potentially severe effects on the success of non-native species management. Examples of such disconnects with negative effects on management programmes are also plentiful (Temple 1990; Wynne 1992; Manchester and Bullock 2000; Bertolino and Genovesi 2003; García-Llorente et al. 2008; Gozlan et al. 2013; Arts et al. 2016; Niemiec et al. 2018; Anderson et al. 2019; Deak et al. 2019; Kochalski et al. 2019; Yletyinen et al. 2021; Kinsley et al. 2024). Discrepancies in views and understanding can be particularly harmful in situations where the wider public can be a significant vector for translocation of non-native species, such as the spreading of non-native fish species through freshwater rivers and lakes by recreational fishers. As such, an understanding of the wider public's perception of non-native species by both managers and scientific researchers is crucial (Nisbet and Scheufele 2009; Verbrugge et al. 2013; Shackleton et al. 2019).

We use semi-structured interviews with managers, researchers and the wider public (in this case recreational fishers) to assess perception of interactions between non-native species and climate change in an area where: a) climate change is progressing at an accelerated rate compared to the rest of the world (IPCC 2021), b) low endemic species richness means the impact of non-native species can carry particular ecological and cultural significance (Hesthagen and Sandlund 2007) and c) the wider public can be a significant vector for translocation of non-native species (García-Díaz et al. 2018; Carpio et al. 2019; Chapman et al. 2020). In exploring said perceptions we aim to identify pathways to integrate scientific, practical and lay knowledge and strengthen collaboration among the three groups. This allows for identification of appropriate management actions to handle these interacting effects of climate change and non-native species (Kapitza et al. 2019).

Methods

Personal interviews

Study system

In order to assess the contrast between perceptions of the interacting effects of climate change and non-native species among three groups - those who produce the scientific research (researchers), those who implement it (managers) and those who provide public approval of its implementation and experience its effects (recreational fishers) - we interviewed respondents from diverse locations throughout Norway in relation to freshwater ecosystems. Norway's location in the sub-Arctic and Arctic, immigration history and topography means that large parts of the country are relatively species-poor and subsequently vulnerable to the effects of non-native species (Hesthagen and Sandlund 2007). Translocations from well before the 1900s until the modern day by various institutions - including the church, the government and recreational fishers from inside and outside of Norway - have resulted in the spreading of native Norwegian species to areas they would not have previously been able to naturally disperse to, as well as the arrival of species non-native to all of Norway and, in some cases, to Europe (Hesthagen and Sandlund 2007; Sandlund and Hesthagen 2011). Many of these species like the pike (Esox lucius) or European perch (Perca fluviatilis) can have negative effects

on native ecosystems and species that are adapted to relatively cold temperatures, many of which are of cultural importance, such as the Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta*) (Winfield et al. 2008; Borgstrøm et al. 2010; Sandlund et al. 2013; Hesthagen et al. 2015; Eloranta et al. 2019).

The increased rate of climate change experienced in the sub-Arctic and Arctic means that, in coming decades, many species that may not have been able to establish and spread through colder ecosystems may be able to do so (Rahel and Olden 2008; Hayden et al. 2017). Effective management of freshwater systems is therefore crucial.

Rotenone treatment of freshwater ecosystems is common throughout Norway to remove harmful non-native species and, while effective, it is expensive and ecologically damaging, so if rotenone treatment is applied, there needs to be assurance that non-native species cannot return easily (Perrin et al. 2020b). Additionally, there is an ongoing trend of dam removal throughout much of Europe, dams which could potentially currently act as dispersal barriers for non-native species (Sun et al. 2020). Norwegians in general consider themselves well-informed regarding threats to aquatic biodiversity relative to other countries and awareness of the danger presented by non-native species is higher than other similar European nations (Falk-Petersen 2014; Kochalski et al. 2019). Crucially, Norwegian fishers are aware of non-native fish species threats and, in some cases, even willing to aid in their removal (Guay et al. 2024). However, the country has seen a lack of agreement between management attitudes and scientific researchers and even within the research community, in regards to non-native species management in the past (Lundberg 2010). This makes consensus in the management of non-native and range-shifting species between researchers, managers and public stakeholders vital in the quest for effective management of Norwegian freshwater ecosystems.

Our study looks at contrasts in perceptions of the interactions between climate change and non-native species throughout Norway. As an explorative study necessitates an understanding of respondents' reasoning, we took a qualitative approach to data collection. There has been a bias towards quantitative methods in similar research in the past, which can limit understanding of the social context in which perceptions are founded (Kapitza et al. 2019). As such, we conducted personal interviews with members of our three chosen key groups; researchers, managers and recreational fishers (Table 1), in line with previous studies (Schüttler et al. 2011; Selge et al. 2011).

Respondent selection

A total of 30 interviews were conducted between August of 2019 and April of 2020. Interview respondents were chosen using the snowball method, as described by Miles and Huberman (1994). This requires an initial pool of contacts, who subsequently nominate other respondents that are suitable for the study. Our initial pool included contacts from a variety of organisations and regions, in order to avoid shared viewpoints potentially based on similar educational and career histories.

In compliance with requirements of the Norwegian National Research Ethics Committee, all respondents were given an overview of the topic beforehand, assured that their responses would be anonymous and informed of the intended use of their responses. Participation was voluntary and respondents could withdraw consent without specifying the reason for doing so. All interviews were anonymously recorded and subsequently transcribed verbatim. Any details which might have allowed the individuals to be identified, based on descriptions of their roles or locations, were removed.

Interest group	Description	Number respondents
Researchers	Professionals associated with public or private research institutes not directly responsible for taking man- agement decisions. Expertise in fish biology or ecology or freshwater ecology or hydrology.	8
Managers	Professionals associated with public organisations which are directly responsible for management deci- sions regarding freshwater bodies.	12
Recreational fishers	Individuals who participate in recreational fishing on a regular or semi-regular basis.	10

Table 1. Description of respondents.

Interview structure

We used a semi-structured interview approach, in order to ensure that interviews flowed as naturally as possible with room for tangential discussions, while ensuring that several basic topics were covered (refer to Suppl. material 1 for interview guide). The first was their perception of a non-native species and whether or not several key factors played into their definition, including: a) method of introduction of the species, b) native habitat of the species and c) societal perception of the species. All three factors have been previously shown to influence perceptions of a non-native species, both among scientific researchers and the public (Warren 2007; Selge et al. 2011). While the English term 'alien species' can be considered as pejorative, it was used in the interview, as it corresponds more accurately to the widely-used Norwegian term 'fremmede art'. So as not to lead respondents into mentioning factors a-c, we asked them to define a non-native species, encouraging them to use examples when needed. We also wanted to gauge whether their view of non-native species changed over time and, if climate change had influenced the species arrival and/or subsequent impact. As recent research has suggested shifting management and research to focus on the impact of non-native species (Jeschke et al. 2014; Wallingford et al. 2020), we wanted to present respondents with a hypothetical situation in which a non-native species established itself and had a demonstrable and reasonably immediate impact, in this case the extirpation of a local species. This hypothetical situation was presented firstly as a result of climate-induced range expansion and, secondly, as a result of human translocation.¹¹ They were asked how they would react to both situations. For fishers, non-native species with which they were familiar were used as an example, in most cases the northern pike (Esox lucius Linnaeus, 1758) or European perch (Perca *fluviatilis* Linnaeus, 1758). They were asked how they would react to both situations.

Additionally, we asked the researchers and managers to name the primary concerns to their region, to capture whether or not non-native species and/or climate change were an acknowledged concern. We also enquired as to which species of fish researchers and managers considered to be of high conservation status. We asked recreational fishers questions relating to their fishing habits, including how long they had been fishing, which regions they had fished in, which species they preferred and whether their preferences changed on a seasonal or longer-term basis. This gave us insight into their perception of particular species.

No time limit was set on the interviews. Interviews lasted anywhere from 10 to 50 minutes, with most interviews taking about 22 minutes. Respondents were invited to talk freely and none expressed discomfort discussing the topic. Respondents occasionally had to be prompted to elaborate on answers in order to better

¹ While every effort was made to assure respondents that the first scenario was hypothetical, two fishers rejected the premise outright, as they felt that introduction of novel species into their local environments was impossible in the absence of human translocation.

understand their reasoning. Although not always relevant, tangents were encouraged in order to allow respondents to better explain opinions or recount experiences. All respondents were offered the opportunity to be interviewed in Norwegian; however, 24 of the 30 were comfortable enough to complete the interview in English. Respondents were encouraged to switch to Norwegian any time they felt unable to adequately express themselves in English. Sixteen interviews were conducted in person, while the remaining 14 were conducted via web meeting. Whether or not the interview was conducted in person did not have a notable effect on the outcome and was, therefore, not used in further analysis.

Response analysis

Responses were categorised, based on two sections of analysis, one of which was common to all groups and one that differed for recreational fishers. The first section analysed which fish species recreational fishers preferred, so as to ascertain whether potential future extirpations would affect the species for which they preferred to fish. We also determined whether or not these preferences had changed over time. For researchers and managers, the first section sought to analyse which species were of high conservation status to their region and for what reasons. We also determined whether or not non-native species and/or climate change were of primary concern and which other factors were considered as primary concerns.

The second section concerned non-native species. We first determined, based on given definitions, whether subjects considered: a) method of introduction, b) societal perception and c) whether the species was native to the part of the country as an important facet of the definition of a non-native species. We then determined whether subjects reacted negatively to the possibility of species extirpations in their local freshwater ecosystems driven by a range-shifting species and whether this response varied when turnover was driven by a non-native species that had been directly translocated by humans. We also determined (although this was not directly elucidated by several respondents) whether or not they thought management action was appropriate in such situations.

In presenting our results, we begin by summarising general findings, then elucidate these findings using quotes from selected respondents. Respondents are referred to by an acronym referring to their respective interest group and order in which they were interviewed. As such, our seventh respondent, a recreational fisher, would be referred to as F-07.²²

Results

The following section will present results, starting with the preferences of recreational fishers, followed by species of conservation concern and local anthropogenic stressors according to managers and researchers. Perceptions of non-native species are then described, followed by reactions to the two hypothetical scenarios.

For the sake of brevity, henceforth the extirpation of local species as driven by range-shifting species will be referred to as climate change-driven turnover.

² Respondent F-04 was in fact three individuals who chose to be interviewed at the same time. As they almost exclusively fished together as a group and responses generally corresponded with one another, their responses were collated into one.

Extirpation of local species driven by non-native species which arrived as a product of direct human translocation will be referred to as translocation-driven turnover.

Extended responses from all respondents are openly available in Perrin et al. (2020c) (https://doi.org/10.5281/zenodo.3991516).

Fishing tendencies

Nearly all fishers interviewed expressed a preference for salmonids, namely brown trout and arctic charr. Several respondents mentioned the value of their preferred species as food fish.

F-18: I went consistently for brown trout since I was a kid, because that's the most common fish in our region. Here, the population of brown trout is dominant in rivers and lakes. It's the most exciting fish to do sportfishing for.

With a few exceptions, these tendencies did not change on any short or longterm basis. Most respondents had fished for their preferred species since they were children. There was some preference for ice-fishing in the winter which restricted fishers to catching charr.

Three respondents also mentioned a dislike of pike and/or perch as a food fish and five specifically stated that they would no longer fish at lakes or rivers where these species had become established.

F-07: I've never fished for pike. But I know lots of people who fish for pike. It's not a good eating fish, like trout is.

Species of high conservation status

Among managers, arctic charr, brown trout and salmon were each mentioned seven times as species of concern. Grayling, eel, pearl mussel, european bullhead, asp, fourhorn sculpin, white bream and vendace were also mentioned. Several admitted that, while they would like to see more focus on the latter species, salmonids were prioritised primarily for economic reasons, although, in some regions, salmonid species were also declining.

M-24: From a biological point of view I guess all species have the same value, from a financial point of view I guess trout and char are the biggest resource...

All eight researchers mentioned at least one salmonid as a species of concern. Burbot, pearl mussels, lampreys, sculpins, cyprinids and notostracan crustaceans were also mentioned.

Local anthropogenic stressors

Non-native species were mentioned as a primary concern to their freshwater ecosystem by five of the eight researchers, with climate change mentioned as a primary concern for six. Eight of the twelve managers mentioned non-native species as a primary concern and eight mentioned climate change.

Perception of alien species

Three of ten fishers mentioned method of introduction in their definition of a non-native species. Respondent F-14 claimed that species that dispersed naturally were non-native, with respondent F-29 feeling that species dispersing naturally were "not necessarily alien" and respondent F-11 claiming that a non-native species "had to be introduced by humans". No fishers mentioned social perception of species in their definition. Two fishers mentioned the species native range, with respondent F-26 defining non-native species as those that are "not native in Norway" and F-05 defining species from the east of Norway as 'unnatural'. All definitions referred generally to fish not belonging in the region or specific lake.

- *F-18:* It means species who aren't originally from that environment. So species you wouldn't have found there originally.
- **F-27:** The definition for me became quite narrow because one of my favourite waters became infected by pike, by some people placing it there because they think it's fun to fish for it. So for me that would be an alien species in that water, it's not supposed to be there.

Seven of the twelve managers mentioned method of introduction in their definition of a non-native species. Of these seven, two definitively named species that spread naturally as non-native species.

M-02: Alien species are primarily those set out by humans. I maybe don't have a clear definition, but if they come here by themselves they can also be alien species.

Two managers stated that non-native species needed direct human help to move.

M-21: I think of course you have had a natural extension and retraction of species always throughout the history of the earth. And of course climate change is affecting this in an unnatural way, but still it's not the same as human transportations of species.

The other three managers did not have a definitive stance either way, but gave impressions on the subject.

M-09: I'm mainly thinking about those who are not spreading by themselves but who are spread by humans. But also those who are coming because of human induced climate change. I think that's not so easy to point out if it's totally alien species or just slightly expanding because of a natural variation.

Three managers mentioned social perception when defining non-native species. Respondent M-20 defined non-native species as something "we don't like", whereas respondents M-24 and M-10 admitted that social perception could influence management approaches to non-native species, though they still classed species as non-native regardless of social perception.

Seven of the twelve managers mentioned whether or not the species was native to Norway as an aspect of the definition. All stated that species which were native to Norway, but not to a local region, should also be classified as non-native in that region. Six of eight researchers mentioned the method of introduction as an aspect of the definition of a non-native species. Of those, four stated that species that moved on their own into new regions were non-native.

SR-17: I think it's a species that's coming to an area where it hasn't been for decades. So it varies, it can come naturally, moving slowly through freshwater species, like some of the alien species we have here that are coming from Sweden.

The other two stated that non-native species needed direct human help to move. Only one researcher mentioned social perception in their definition, with respondent SR-25 claiming the definition was "value-based". Two researchers included whether or not the species was native to Norway in their definition, with both stating that species native to a certain region of Norway could still be classified as non-native in other areas.

SR-19: I know when we use this term we need to specify if we mean truly alien, like not even belonging in this country, or just having moved to a new area. But for me they mean both...

Perception of climate change-driven versus translocation-driven turnover

All fishers felt negatively about climate change-driven turnover, with all citing their inability to fish for their preferred species as the main reason. Several used strong or emotive language in their reaction to the hypothetical scenario.

*F-18: F**** off. Would be my answer. It would be a terrible situation for my passion. It's that easy. I don't have a big interest in dry fly fishing for perch or pike.

Only one respondent mentioned ramifications for the local ecosystem as a contributing factor to his reaction. Several respondents recognised that climate change can make lakes more suitable for other species, but that these lakes should still be preserved.

F-27: That would feel bad, it would ruin my waters. I wouldn't like that, and I think we should try to prevent it, even though it's climate change, we should stop those things from happening.

There was no inversion of response when asked how they felt about translocation-driven turnover; however, four felt even more negatively about this possibility.

F-14: I think I would get more angry if it was humans. But I wouldn't be happy either if it was climate change. People should know... the consequences of moving species over.

While some fishers did feel negatively about the prospect of climate change-driven turnover, they felt it was unlikely to occur in their local ecosystems in the near future. Nine of twelve managers felt negatively about climate change-driven turnover. Three of those managers cited potential effects on local fishers as a contributing factor to their reactions. Of the nine, only four felt that management steps should be taken to prevent non-native species from establishing in lakes as a result of range-shifts.

M-22: ...some species will spread, even though they're alien species, because you simply don't have the possibility to stop them. But in other respects, I would resent or try to stop such a development... Because you also have to bear in mind that these are alien species and you should give the native species a possibility to adapt from climate change...

Of the managers who did not feel that management actions were warranted in the case of climate change-driven turnover, most stated that they felt it was futile to combat long-term changes.

M-21: ...it's a result of a new climate situation, and it's not possible to try to fight this I think. I think the species living in the environment has just adapted, and we lose some and we get some... It's not possible to try to maintain the status quo if the climate changes.

The manager who did not feel negatively about climate change-driven turnover, respondent M-01, also did not feel negatively about translocation-driven turnover, stating that, as their region of concern did not have any incoming non-native species of concern, no action would be needed.

Five of the nine managers who felt negatively about climate change-driven turnover stated that they would feel more negatively about translocation-driven turnover.

M-24: I think then I could direct, my anger, my mood I guess, my emotions would be directed. More disappointment and anger, those kinds of feelings I guess. We would have to look at how this was allowed to happen, and adapt a management scheme to it I guess.

Of the five managers who felt negatively yet did not feel that management steps should be taken to mediate climate change-driven turnover, four felt that management steps would be warranted in cases of translocation-driven turnover, with one explicitly stating that they had performed management actions in such cases.

M-21: If a species is moved by humans into a new area we will actively try to remove it again. We have a lot of examples of that, we've spent money on that. It's very difficult to succeed with such an approach, but we do it.

Four of the eight researchers did not feel negatively about the possibility of climate change-driven turnover, with many arguing it was a natural process.

SR-25: If for some reason a new species is able to survive in an area now that it couldn't before, I think that's life. And to put a lot of management efforts into avoiding that, I think that's a bad solution. There are so many other things to use limited resources on.

Four researchers felt negatively about the process, but two did not think that management was warranted as it would be futile.

SR-19: I would also feel that it was nothing we could do, and accept it, and try to focus on something else... because it would be very difficult to artificially keep other species alive in systems which isn't suitable for them any more.

All researchers had a negative opinion concerning the prospect of translocation-driven turnover. Of the six who did not think that management action should be taken to avoid climate change-driven turnover, all six expressed that it was appropriate to combat translocation-driven turnover.

SR-16: ... obviously if there is a human introduction, then I would view that more negatively ... with human induced temperature increase, that would be a pretty strong concern, but then with a direct introduction, that would be even more of a concern, because we have the knowledge, to know that we shouldn't really do that, that that will mess up the natural ecosystems.

Discussion

Ensuring that there is correlation between the views of scientific researchers, managers and the general public is critical when implementing conservation strategies. This is especially the case when the strategies involve complex and controversial subjects, such as the interacting effects of non-native species and climate change (Pecl et al. 2017). Here, we aimed to identify possible causes of disconnect between these different groups in their perceptions of the interacting nature of climate change and non-native species and their subsequent impact on freshwater ecosystems. Our analysis shows that attitudes vary within and between managers and researchers to the impacts of non-native species when they are, in part, driven by climate change. However, the same impacts are almost unanimously negatively viewed by a public group – in this case recreational fishers – with the impact of climate change on the introduction of the non-native species having very little effect on their opinions.

The most prominent contrast between the groups was the fishers' response to climate change-driven turnover compared to that of the managers and researchers. While there were conflicting feelings about climate change-driven turnover among the managers and researchers, the prospect was unanimously rejected by recreational fishers. Although some admitted they would be angrier if human translocation were the sole culprit, many stated that they would view the presence of a non-native species and/or the loss of native species negatively, regardless of whether or not climate change had influenced the outcome. Many felt that management action should be taken to prevent such turnover wherever possible. This lack of consensus between groups is not unexpected, as instances in which there are disagreements between local stakeholders who are directly impacted and managers and researchers are far from uncommon (Redpath et al. 2013; Manjarrez-Bringas et al. 2018).

Contrast in the impacts and management of non-native species and climate change was present within groups as well, most notably among managers and scientific researchers. While most expressed negative opinions about the process, there was a variety of opinions in both groups regarding whether or not management action should be taken. While some supported removal, many found it to be futile – even in cases where lack of removal would result in a local extirpation –

while others thought it would be unwarranted even if removal were possible. This is unsurprising, as dialogue regarding the concept of range-shifting species is often polarised (Shackelford et al. 2013). However, it does suggest a lack of consensus on a management issue that may become more pressing in the coming decades.

The unanimous rejection of new species by fishers was often mentioned in conjunction with the new species having little or no perceived value as a food resource. Further investigation into how heavily the value of a species as a food resource factors into public perception of a species is warranted, including whether perception would shift if the incoming species had more in common with preferred species, such as the previously introduced species brook or lake trout. Familiarity with a species has previously been shown to affect public perception of them as non-native or not (Kochalski et al. 2019) and emotion can often play a larger role than rationale in shaping opinions on fish as a food resource (Verbeke et al. 2007). While pike does not appear to be a preferred food-fish in Norway, it is well-regarded elsewhere in Europe (Linhart et al. 2002). Qualitative studies in areas where species have been established for longer periods of time may shed more light on the role of the public's familiarity with non-native species in their reaction to them.

Similar contrasts in the perception of climate change-driven turnover are evident in the varying definitions of non-native species across the different groups. While it featured in the definitions of over half both the managers and researchers, method of introduction was generally not addressed by the fishers in their definition of non-native species. Furthermore, although several fishers acknowledged that climate change would likely alter nearby ecosystems, only one alluded to the possibility of new species arriving. This could be a result of a lack of knowledge regarding the effects of range shifts as a product of climate change or an association of non-native species as primarily being a product of human translocation.

Given the global restructuring of ecosystems that is currently taking place as a product of climate change gradually altering species ranges, more open communication among all three groups should be a priority in ecosystem management. Going forward, perhaps the most notable area of disconnect between the groups is the question of whether management actions should be taken to prevent the impacts of non-native species, even when such impacts are driven by climate change. The reluctance to commit resources to stop such impacts among managers and researchers compared to the insistence that such management was required by the fishers represents the most obvious source of potential future conflict identified in this study. Previous research in marine systems has suggested that fishers do not tend to automatically link climate change to the arrival of new species (van Putten et al. 2016) and that educating public stakeholders is crucial to the success of future policy regarding climate change and range-shifts (Nursey-Bray et al. 2012; Pecl et al. 2017).

More open communication among the three groups, particularly between scientific researchers and public stakeholders, will be key to increasing support for management actions in the future (Courchamp et al. 2017). To ensure uptake and support for management actions, the scientific research that drives them needs to be trusted by recreational anglers (Wynne 1992; Weyl et al. 2014). Both national and more local managers have a key role to play in building this trust, as they can often sit as the intermediary between the two groups. Local managers possess valuable local knowledge applicable to different regions, which can ensure better knowledge transfer (Nisbet and Scheufele 2009), presumably resulting in increased public understanding of invasive species' effects and more support for their management (Lindemann-Matthies 2016). Managers, particularly those in national bodies, can also facilitate the formation of collaborative networks across regions, ensuring both more cooperative interaction between affected regions and more standardised messaging, both of which can increase the efficacy of management policy (Courchamp et al. 2017; Niemiec et al. 2018; Kinsley et al. 2024).

The facilitation of regular workshops involving all three groups would enable direct dissemination of scientific research to anglers, but also allow researchers to better understand both management challenges and the public's perception of their research (Shackleton et al. 2019). Such workshops would also provide an opportunity to include recreational anglers in management projects from the outset, a tactic which often results in higher public uptake of management initiatives (García-Llorente et al. 2008; Weyl et al. 2014; Pecl et al. 2017; Novoa et al. 2018). A bonus would be training in scientific communication among scientific researchers, as effective communication requires framing scientific research for specific target audiences (Nisbet and Scheufele 2009). Repeated interactions between researchers, managers and the public also have the potential to build trust and increase the acceptance of management decisions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

In compliance with requirements of the Norwegian National Research Ethics Committee, all respondents were given an overview of the topic beforehand, assured that their responses would be anonymous and informed of the intended use of their responses. Participation was voluntary and respondents could withdraw consent without specifying the reason for doing so. All interviews were anonymously recorded and subsequently transcribed verbatim. Any details which might have allowed the individuals to be identified, based on descriptions of their roles or locations, were removed from the transcriptions.

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Author contributions

SP and CL conceived the idea. SWP, CPW and CL designed the methodology. SP and AGF sourced the initial pool of respondents. SP collected and analysed the data. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability

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Supplementary material 1

Interview guide

Authors: Sam Wenaas Perrin, Carina Lundmark, Camilla Perrin Wenaas, Anders Gravbrøt Finstad Data type: pdf

- Explanation note: A guide used for semi-structured interviews to assess perception of interactions between non-native species and climate change in Norwegian freshwater ecosystems.
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Research Article

Long-term seed survival of common ragweed (*Ambrosia artemisiifolia* L.) after burial

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Abstract

Ambrosia artemisiifolia is a serious threat to human health and agricultural yield. Due to its annual growth form management should focus on the prevention of seed production in the long run. The long-term survival of ragweed seeds depends on the implementation of viable seeds to the persistent soil seed bank. In a field study, we tried to find out how long this species must be surveyed/managed to reach the goal of complete eradication after burial of seeds into mineral soil. We tested for the influence of different seed sources (origin), different soil depths of burial, different experimental sites in Middle Europe (labs), and duration of burial on the viability of seeds by germination test plus TTCtest. In our study, seed origin had a highly significant influence on the seed survival. In all the 10 years of the experiment, seeds sampled from a rural stand in Austria showed significantly lower viability rates than seeds from Hungary. The Hungarian seeds from arable fields had viability rates of up to 90% even after 10 years' burial. Burial depth (7 cm/25 cm) had no significant influence on the viability rates but we detected a serious influence of the experimental sites which can be caused either by the burial site conditions (differences in soil and climate) or by different implementation of the manuals for germination tests and colouration test using 2,3,5-triphenyltetrazolium chloride. The decline of viability within the 10-year period differed by seed origin, but was generally faster in the first few years but relatively low in the following years. Due to the fact that we found 30 to 90% viable seeds after 10 years burial there is substantial evidence that soil perturbation (digging animals, ploughing) should be avoided for even more than ten years in habitats that are highly infested with ragweed.

Key words: Control measures, dormancy, germination, invasive species, soil seed bank, weed management

Introduction

Common ragweed (*Ambrosia artemisiifolia* L.) is considered one of the most dangerous invasive alien weed species in Europe (Smith et al. 2013; Essl et al. 2015). First of all, its pollen causes allergic diseases in humans, inducing enormous costs due to resultant necessary medical care and sick-leaves (Schindler et al. 2015; Schaffner et al. 2020). Furthermore, ragweed causes substantial yield losses in several crops (Pimentel et al. 2005; Soliman et al. 2010; Novák et al. 2022), and alters



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the competitive balance in rare weed communities (Pál 2004) and some species rich grasslands (Karrer et al. 2011). Many projects were initiated by national and EU authorities that aimed at developing measures against ragweed (Buttenschøn et al. 2009; Karrer et al. 2011; Bullock et al. 2012; Söltner et al. 2016a; Müller-Schärer et al. 2018). A leaf feeding beetle (*Ophraella communa* Lesage) from the native region of ragweed got established in some parts of Europe (Bonini et al. 2015), something that might help greatly to overcome the problems caused by this dangerous weed (Augustinus et al. 2020; Schaffner et al. 2020; Keszthelyi et al. 2023).

The invasiveness of common ragweed was documented for Europe but also for other continents, i.e., Asia (Watanabe et al. 2002; Qin et al. 2014) and Australia (Parsons and Cuthbertson 2001). Both mechanical and chemical measures are able to reduce the aboveground populations of ragweed (Kazinczi et al. 2008; Buttenschøn et al. 2009; Söltner et al. 2016a). Nevertheless, in the case of long-time persisting populations ragweed recovers easily from the soil seed bank because the seeds can survive in the soil for up to 40 years. Toole and Brown (1946) documented that at least a few seeds were able to germinate after this long period of subterranean dormancy. Considering that one single individual of common ragweed is able to produce up to 94.000 seeds (Kazinczi et al. 2008) and these seeds are commonly installed in deeper soil horizons by ploughing, ragweed develops to a nasty weed in many crops, i. e., summer crops like maize, sugar beet, sunflower, and soybean (Kazinczi and Novák 2014; Karrer 2014).

In agroecosystems the average seed production is around 4000 achenes (Kazinczi et al. 2008) for one plant individual. The higher the plant biomass, the higher the seed yield (Lommen et al. 2018). The infestation of the arable soil weed seed bank with ragweed seeds is very high in Hungary and Austria (Kazinczi et al. 2008; Karrer et al. 2011) and was even enhanced during the last decade (Kazinczi and Pál-Fám 2018), ensuring long-term ragweed infestation in the cultivated areas.

Long-term experiments showed that the survival rates of weed seeds under field conditions were best in deeper soil layers. Toole and Brown (1946) found 6% of ragweed seeds still germinable after burial for 39 years at a depth of 22 cm. At least 21% and 57% of seeds buried in the soil at 8 cm and 22 cm, respectively, germinated 30 years after the start of the experiment.

However, ragweed seeds deposited on the soil surface under field conditions were found viable for 4 only years (Beres 2003; Kazinczi et al. 2011). Kazinczi et al. (2011) reported viability rates of 18% when ragweed seeds were stored under dry conditions at room temperature (20 °C) after 5 years. Intraspecific differences regarding germination characteristics of common ragweed are also known (Kazinczi et al. 2006; Onen et al. 2020). After 7, 6 and 4 years of dry storage, ragweed seed viability was 15, 45, and 72%, respectively (Kazinczi et al. 2011). After three years of storage, viability rates of ragweed seeds varied between 62 and 90%, depending on the origin of the tested populations (Kazinczi and Kerepesi 2016).

Soil perturbation by ploughing incorporates weed seeds like those of ragweed to deeper soil horizons where weed seed survival rates increase with burial depth (Froud-Williams et al. 1984). We know the maximum age of buried ragweed seeds from the Durvel burial experiment (Toole and Brown 1946) but there is still a lack of knowledge about the annual decrease of seed viability during the first years of burial. Therefore, a long-time experiment was initiated within the framework of the EU-project HALT Ambrosia (https://ojs.openagrar.de/index.php/JKA/article/view/1792) to test annually the ragweed seed viability buried at different soil

depths for a period of one to ten years (Karrer 2016a, Karrer et al. 2016a). The main aim was to find out the annual decrease of seed viability in general but also germinability as major part of the viability.

Materials and methods

Two populations of ragweed seeds were sampled in autumn 2011 from arable fields in Hungary (Kaposvár: 46.368608, 17.851789; 148 m a.s.l.) and from ruderal arable fields in Austria (Hagenbrunn: 48.343333, 16.466278; 178 m a.s.l.). Seeds were air dried and stored at room temperature (\pm 20 °C).

Field experiments were established in the experimental farm areas of Kaposvár University (Kaposvár: 46.368608, 17.851789; 148 m a.s.l.) and of BOKU University (Groß-Enzersdorf: 48.199417, 16.557611; 154 m a.s.l.). Seeds were buried in winter 2011/2012 at two soil depths (upper layer (5–8 cm), and lower soil layer (25 cm).

In a pre-trial, another seed lot from Austria (Styria, Unterpurkla: 46.731500, 15.901528; 229 m a.s.l.) was sampled in 2010 and buried in the botanical garden of the BOKU University (Vienna: 48.237194, 16.332361; 236 m a.s.l.) in winter 2010/2011 at a soil depth of about 10 cm. This pre-trial gave us some valuable technical and practical experiences for the main trial.

Portions of 50 seeds were enclosed each in polyester mash before burial (Fig. 1, left). For the main trial, we buried 50 bags with 50 seeds each at the two different soil depths in Kaposvár and Groß-Enzersdorf, and for the pre-trial 70 bags with 50 seeds each in Vienna.

The buried seed lots represent spatially independent replicates (Fig. 1).

Excavation of the seeds of the pre-trial (7 bags each year) started in 2012 and ended by 2021. In the main experiment, excavation of 10 bags per year (5 from each layer) ranged from the year 2013 until 2022. Excavated seed bags were transferred to the lab for immediate germination tests.

Seed viability was tested by both labs following the manuals of Karrer et al. (2016a) and Starfinger and Karrer (2016). First step: germinability was tested directly after excavation. Due to the fact that the seeds were buried for one year minimum in the soil under field conditions their need for vernalisation (Willemsen 1975; Bassett and Crompton 1975) was adequately met. Intact seeds from every bag were put into petri dishes on moistened filter paper and placed for 4 weeks into climate chambers running a cycle of 12 hours light at 30 °C and 12 hours darkness at 15 °C (Leiblein-Wild et al. 2014; Karrer et al. 2016a, 2016c). Every second day the number of germinated seeds was counted and removed. A seed was stated "germinated"



Figure 1. Seed bags (left), prepared for burial (right).

when the radicula was visible at a length of 2-3 mm. Second step: All seeds that did not germinate (probably because of dormancy) were subjected to a standard viability test of the embryo. For this TTC-test we used 2,3,5-triphenyltetrazolium chloride to induce red colouration of the living cells of the ragweed embryo. The TTC-test procedure followed Starfinger and Karrer (2016) and Hall et al. (2021). The seeds were already in a soaked condition after the germination test and immediately cut with a medical scalpel longitudinally into two halves to check the constitution of the embryo using a microscope. Dead embryos (decomposed or degraded in the sense of Hall et al. 2021) and empty seed coates were classified non-viable before the TTC-test started. Apparently intact embryos were put into 0.5 ml PCR-tubes that were filled with 1%-TTC solution (powder dissolved in demineralised water) and incubated at 30 °C for 24 hours in darkness. Then the embryos were checked for discolouration using a microscope. The discolouration of the embryo was classified to three types (Suppl. material 1: fig. S1): (a) fully stained in red (= viable), (b) partially stained red or orange (intermediate) and (c) not stained at all (dead), following the protocol by Starfinger and Karrer (2016). Intermediate discolouration was always connected with non-coloured radicula. This inactive (= dead) radicula disables the embryo to break through the seed coat for successful germination. Therefore, intermediates were counted as non-viable seeds for statistics (Hall et al. 2021).

Before burial the sampled seeds from 2010 and 2011 were also tested in Vienna for germinability, and viability by TTC-test, after 6 weeks of vernalisation in darkness at 4 $^{\circ}$ C (n = 100 each seed lot).

In parallel to the burial trial, seeds from the Kaposvár seed lots were stored for 10 years in dry conditions at room temperature and tested for germinability and viability annually following the same procedure as administered to the buried seeds.

Analysis of the data were performed either on the number of germinated seeds, or on the number of TTC-positive seeds, or on the number of viable seeds from both subsequent tests. "Viable" seeds comprise therefore finally the germinated seeds plus TTC-positive seeds from the TTC-test that was applied to the non-germinated seeds. Statistical analysis of germinability and final viability was applied to arcsin-transformed data. GLMM and ANOVA was used to describe differences of viability with respect to the independent factors 'seed origin', 'burial depth', 'burial site' (lab, resp.) and 'year of excavation'. For fine-tuning the results of multiple regression analysis we constructed generalised linear mixed models (GLMM) using R packages lme4 (Bates et al. 2018), MuMin (Barton 2018), and AICcmodavg (Mazerolle 2017) aiming to detect the best model (factor combination) that can explain the viability of seeds. Collinearity of the explanatory variables "burial site", "seed origin", "burial depth", and "year of excavation" was tested using R-package corrplot (Wei et al. 2017), and could be precluded. Models were selected by comparing the second order Akaike Information Criterion value (AICc value) corrected for small sample sizes. To identify the most parsimonious model based on the lowest AICc value we computed the AICc differences (Δ AICc) between the different candidate models. As a rough rule Burnham and Anderson (2002) proposed that models for which $\Delta i \ge 2$ receive substantial support as the chance of the smaller AICc value being correct lies at approx. 73%. Group differences considering the year of excavation were post-hoc tested by Tukey HSD. Other groups defined by binomial factors were checked for significant differences of their means at a significancy level of p < 0.05 by non-parametric tests (Kruskal-Wallis-Test). The homogeneity of variances was tested with Levene's test. The correlation of germinability and viability-rates was tested by Spearman' Rank Correlation.

Results

As ragweed seeds are known to stay dormant under specific conditions we tested the initial germinability and viability rates of the seed lots before burial. The seeds from Unterpurkla used in the pre-trial in Vienna were germinable at an average of 72% and finally viable (germinated and TTC-positive) at an average of 82%, before burial. Pre-burial-tests of the seed lots from Hagenbrunn and Kaposvár in the lab in Vienna gave 14% and 76% germinability, and 18% and 85% viability, respectively. In general, the seeds started with less than 100% viability due to some dead embryos, which could not be detected from outside the obviously intact seeds.

Pre-trial in Vienna

For the trial at the BOKU-garden in Vienna with seeds from Unterpurkla, excavated seeds started with very low mean viability rates of 43% after the first year of burial (2012). After two years' burial seed viability was measured at 73% which was about the same values as the seeds before burial (2011: 72%). In subsequent years the viability rates dropped to a level of \geq 40%. Only in the very last year (2021) ragweed seeds showed a marked further viability decrease to 30%. (Fig. 2). The low viability in the year 2012 seems to be accidental.

Main burial experiment

The trial gave results for germination rates as well as for total viability rates (including TTC-positive seeds). Viability rates are generally equal to or higher than the germination rates. Both rates are positively correlated (R = 0.884, p < 0.001). But there is a difference between the places of burial/analysing labs. The germinability rates were almost as high as the viability rates and reached a perfect linear correlation for the Austrian labs whereas for the Hungarian data the linear regression coefficient was less positive but nevertheless significant (Suppl. material 1: fig. S2).

In the main burial experiment four influential factors on germinability and viability were tested.





The ANOVA with all four factors showed a significant influence of 'seed origin', 'year of excavation', and 'burial site/laboratory' (p < 0.001) on the viability rates of buried ragweed seeds (Suppl. material 1: table S1). The factor 'burial depth' had significant effect on viability in the four-factorial analysis but not in the one-factorial ANOVA (F = 0.674, p = 0.350).

The GLMM analysis started with the calculation of the explanatory power of each stand-alone factor, indicating that seed viability was particularly affected by the factor "seed origin". As null model we used the factor "burial site/laboratory" to test if results are influenced by site specific conditions and/or lab conditions but this could be mainly excluded. However, when calculating the models, it became obvious that seed viability of common ragweed was mainly explained by the interaction of the factors "seed origin", "year of excavation" and "burial site", indicating that there is some influence of site/lab specific conditions. Additive effects of the factors showed only very low AICc values (not shown in Table 1), and can therefore be excluded. Furthermore, in contrast to the four-factorial ANOVA analysis the factor "burial depth" had almost no explanatory power over the results.

Years of excavation significantly affected seed viability rates in general (Fig. 3; overall ANOVA: F = 4.222, p < 0.001), and specifically, the years 2020, 2021 and 2022 had significant pairwise differences compared to three or more years before at the level of p < 0.001.

Table 1. Summary of AICc values used for model selection of dependent variable seed survival rate; number of estimated explanatory parameters and parameter combinations = 8; AICc = Second order Akaike Information Criterion; Δ AICc = difference between AICc to the next most parsimonious model; R² = proportion of variance explained by the factors on the (arcsin-transformed) viability rates of buried ragweed seeds.

	Explanatory model	AICc	ΔAICc	\mathbf{R}^2
Viability of seeds	Null Model: Burial site (Laboratory)	433.5		
	Seed origin * Year of excavation * Burial site	-206.2	0.0	0.86
	Seed origin * Year	-45.2	161.0	0.81



Figure 3. Seed viability rate (box-plots, percentages of viable ragweed seeds per bag) from 2013 to 2022.

In general, seeds originating from Kaposvár/Hungary had significantly higher viability rates than the seeds from Hagenbrunn/Austria (means at 89.45 and 47.92, resp.; Mann-Whitney-U-Test: p < 0.001). This significant difference in viability rates was detected for all years (evident from Fig. 4).

Interestingly, the burial site/testing lab showed also significant influence on the viability rates of ragweed seeds (Fig. 5, Suppl. material 1: table S1). In the first 6 years of the experiment the mean viability rates of buried ragweed seeds differed significantly between the burial site/lab. From the 7th year onwards, the viability measures by the different labs (burial sites) were almost identical.

When seed viability rates were compared with respect to year of excavation and burial depth no significant difference was found (F = 1.478; p = 0.155, Fig. 6).

But when the data are presented in groups by seed origin and burial site (Fig. 7), viability rates differ significantly between all compared groups at p < 0.001 (Krus-kal-Wallis-Test, two-tailed). Along the years of excavation both seed lots decreased in viability rates in both places of burial, but the Hagenbrunn seeds lost viability



Figure 4. Seed viability rate (box-plots, percentages of viable ragweed seeds per bag) from 2013 to 2022 with respect to seed origin.



Figure 5. Seed viability rate (box-plots, percentages of viable ragweed seeds per bag) from 2013 to 2022 with respect to burial site (AT = Groß-Enzersdorf in Austria, HU = Kaposvár in Hungary).



Figure 6. Seed viability rate (box-plots, percentages of viable ragweed seeds per bag) from 2013 to 2022 with respect to burial depth of seeds.



Figure 7. Seed viability rate (box-plots, percentages of viable ragweed seeds per bag) with respect to seed origin (Ha=Hagenbrunn/Ka=Kaposvár) and burial site (AT=Austria/HU=Hungary); letters correspond to significant group differences in means.

from the first years onwards with a very low starting value when buried in Austria, whereas the same seed lot buried in Hungary start their loss of viability from a far higher level (Fig. 8, Suppl. material 1: fig. S3, table S3); only in the last three years, their viability dropped almost to the level of Hagenbrunn seeds buried in Austria. The Kaposvár seeds lost barely no viability when buried in Hungary (> 95%). Only in the last year (2022) viability dropped to a mean of less than 90%. When the same seed lots were buried in Austria we found more than 90% viability in the first three years only. The overall means for the Kaposvár seeds dropped slowly but not significantly to just above 90% viability until 2020. In 2021 and 2022, the mean viability of Kaposvár seeds buried in Austria dropped to 89 and 83%.

Discussion

Seeds of common ragweed are known to stay dormant when the conditions (i.e., burial in deeper soil horizons, long dry periods, missing stratification by several



Figure 8. Seed viability rate (box-plots, percentages of viable ragweed seeds per bag) from 2013 to 2022 with respect to factor combinations of seed origin (Hagenbrunn/Kaposvár) and burial site (\mathbf{A} = Austria / \mathbf{H} = Hungary).

weeks of low temperature after seed ripening) do not allow germination (Dickerson 1968; Willemsen 1975; Bazzaz 1979). In earlier experiments, Dickerson (1968), Kazinczi et al. (2008), and Farooq et al. (2019) found reduced germinability when seeds were buried at six to eight centimeters soil depth. It can be expected that those seeds that are buried in even deeper soil horizons are not stimulated to germinate and stay in enforced dormancy over years. But long-time burial cannot hold the viability of buried seeds of any kind of weeds at high levels. In the case of common ragweed, Duvel started a burial experiment of several weedy species in 1902 to determine seed longevity under natural conditions. Maximum age for survival was 39 years (Toole and Brown 1946) for seeds that were buried in depths of 56 cm (germination rate at 6%) and 106 cm (22%). In our experiment we tested germinability and final viability of ragweed seeds buried in two different soil depths. In general, germinability and overall viability rates differed slightly but not significantly. Only in some years and exclusively at the Hungarian burial site, the seeds buried in deeper soil showed higher germinability and viability rates than those from shallower soil depths (Suppl. material 1: table S4).

Viability rates comprised of germinated seeds and subsequent TTC-test on the non-germinated seeds. Therefore, germination rates were somehow lower than rates of viability, but the burial and test conditions in Hungary differed obviously to those in Austria. This confirmed the fact that all germinating seeds are viable but not all viable seeds germinate – due to seed dormancy – similar to the majority of any weed seeds (Baskin and Baskin 1980, 1998).

Our experimental design did not allow to clarify the role of potential factors that may cause some of the differences in the viability results. Climatic conditions during burial (Suppl. material 1: table S5) might have some influence but we found no interpretable differences. Some influence in the viability results may be caused by differences in the seed manipulations during the experiment (conditions of excavation and transportation to the lab) or by differences in the interpretation of colouration of seeds by the TTC-test. As long as human interfaces are involved in the interpretation of TTC-colouration of seeds, this may cause images under discussion (Zhao et al. 2010; Busso et al. 2015; Hall et al. 2021). In fact, Austrian TTC-positive seeds were few and did not raise the total viability rates this much compared to Hungary. Possibly, the Hungarian lab classified less intensive stained seeds as not viable in some cases.

Viability values of the pre-trial in Vienna indicated a significant decrease in the first 3 years from 72% to about 40%. In subsequent years there was no further loss of viability except for the last year (30%). This result runs contrary to the main experiment results (Fig. 8) where specifically the seeds originating from Kaposvár started at very high viability rates of more than 95%. Even after 10 years, the viability rates of buried seeds originating from Kaposvár were reduced only to 85% in the Hungarian lab, and to 70% in the Austrian lab. The seeds originating from Hagenbrunn in Austria started in the Hungarian lab also with relatively high viability rates of around 90% and dropped drastically only in the last three years to 35%. On the other hand, the same seed lots buried and analyzed in the Austrian lab started in the first year with only 30% viability and dropped slowly to less than 20%. Burial depth had no significant influence on these interesting differences.

Several factors may influence the germinability and viability in burial experiments like ours: When seeds are sampled in the field seed sizes and ripening stage may vary to some extent. The conditions of storage or transportation of weed seeds may have an influence on their viability (Moravcová et al. 2006; Karrer 2016b, Kazinczi and Kerepesi 2016; Starfinger and Söltner 2016), i. e. when the seeds differ already in size, weight or ripening stage (Karrer et al. 2012; Karrer 2016c; Söltner et al. 2016b). Furthermore, the soil conditions (dynamics and range of soil temperature and moisture) at the place of burial might have an influence on seed survival (Farooq et al. 2019; Nikolić et al. 2020).

Obviously, the burial of ragweed seeds at seven or more centimetres of soil depth is deep enough to stop initiation of germination. We found only a slightly higher (but not significant viability of seeds buried deeper into the soil at 25 cm. This is in line with the results of Toole and Brown (1946) who found also higher survival rates of ragweed seeds buried at about one metre in the soil. Benvenuti et al. (2001) found that typical annual weeds of arable fields did not germinate in depths of \geq 6 cm. This seems to hold also for our annual weed species. Other weeds like Echinochloa crus-galli showed also sensibility of age and storage conditions of seeds. Moravcová et al. (2022) found that storage of barnyard grass at room temperature ended in lower germination rates and the loss of germinability within 2 years whereas seeds buried for 8 years in 20 cm soil depth showed germination rates of 40-60%. In the soil the seeds experienced several dormancy/non-dormancy periods without germination and could stay germinable for longer time. We tested also the germinability and viability of seeds of the different origins stored either at room temperature (Hungary) or at 4 °C in darkness (Austria) and found that the Kaposvár seeds stored at room temperature lost viability continuously (2013: 96%, 2014: 96%, 2015: 80%. 2016: 47%, 2017: 23%, 2018: 13%, and 2019 onwards: 0%). The Hagenbrunn seeds stored in darkness and at 4 °C in Vienna were measured with lower and quickly decreasing viability levels; i. e., 2013: 54%,

2014: 14%, 2015: 12%, 2016 onwards: 0%. Obviously, the 'seed quality' of Kaposvàr seeds was far better than those provided from Hagenbrunn/Austria. This is astonishing as the average seed weight of the Hagenbrunn seeds was significantly higher than in the Kaposvár seeds: means (n = 200, each) 5.37, sd \pm 1.89 mg and 3.59 ± 1.29 mg, respectively. The seeds for the pre-trial (from Unterpurkla) were measured with 4.01 \pm 1.66 mg. In our experiments, the smaller the seeds, the better the germination performance and viability. Similar results were found in the tree *Copaifera langsdorffii*, where smaller seeds germinated quicker than big ones and invested more to the root system (Souza and Fagundes 2014). Following these authors, this behaviour can be interpreted as adaptation to the colonisation of transient habitats and early successional stages like arable fields (Baskin and Baskin 1998). Kazinczi and Kerepesi (2016) showed in earlier experiments that seed age and origin greatly influenced seed viability.

Our results confirmed the fact that under field conditions ragweed seeds can remain viable for a long time, especially in the deeper soil layers, so its seeds can enrich the persistent soil seed bank in habitats with regular soil perturbance as in arable fields. Seeds that are deposited on the soil surface or beneath shallow litter layers undergo the dormancy/non-dormancy environmental influences in temperate regions every year. They are prone to germinate easily due to nice water and light supply after having experienced break of dormancy until early spring. In such populations the soil seed bank is lower in numbers of viable seeds (Karrer et al. 2011).

In our experiment it became evident that the exactness of sticking to the experimental protocols is essential to gain comparable data. Starfinger et al. (2012), Karrer et al. (2016b) and Hall et al. (2021) used ragweed germination and viability testing data to show the great influence of lab conditions and discipline, circumstances of sampling and storage of seeds and environmental conditions of burial sites, as well as the rules for interpretation of primary viability results (colouration of seeds in TTC-tests).

So from the point of integrated weed management in arable fields we suggest to prefer preventive control procedures, primarily to prevent the flowering and seed production of ragweed (Karrer et al. 2011; Kazinczi and Novák 2014; Karrer 2016d). This represents a long- term control strategy in terms of reducing ragweed populations.

To conclude, we found significant differences in the viability of ragweed seeds with respect to seed origin which was interestingly negatively correlated to seed weight. Effects of burial site/lab conditions were also significantly different over the whole duration of the experiment, but towards the end (after 8 years) the differences collapsed. Our tested soil depths had no significant influence on viability, indicating that burial at ± 7 cm fulfils the need of ragweed seeds for continuation of innate dormancy. The loss of viability with ageing of buried seeds was expected although this effect was less prominent in the Hungarian seed origin. Hungarian seed populations from Kaposvár experienced a far longer period, by about 70 years, of successful invasion and establishment of common ragweed compared to the Austrian population with about 10 years. This might have promoted the development of a segetal weed population with prolonged buried seed survival rates adapted to the local agricultural regimes in Hungary whereas the ruderal population in Austria was far younger and still not adapted so much to burial processes.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualisation, resources, project administration and supervision: GKAR, GKAZ. Formal analysis: GKAR, GKAZ, RH, JP. Investigation: GKAR, FL, NW, RH, BK, JP, IJ. Methodology: GKAR, GKAZ. Writing – original draft: GKAR, GKAZ. Writing – review and editing: GKAR, GKAZ, RH.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary data

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Data type: pdf

- Explanation note: **figure S1.** Discolouration and viability stage after TTC-test of common ragweed seeds; **figure S2.** Linear correlation of germinability and total viability of ragweed seeds buried in Austria and in Hungary; **figure S3.** Synoptic boxplots of viability rates of ragweed seeds with respect to seed origin, burial site/lab, year of excavation, and grouped by burial depth; **table S1.** ANOVA results about the influence of the factors seed origin, year of excavation, place of burial/ lab, and burial depth on the viability rates of buried ragweed seeds; **table S2.** ANOVA results about the influence of the factors seed origin, year of excavation, place of burial/ lab, and burial depth on the viability rates of origin, year of excavation, place of burial/ lab, and burial depth on the germination rates of buried ragweed seeds; **table S3.** Viability rates of ragweed seed lots originating from Hagenbrunn or Kaposvár, buried in Austria or Hungary and excavated from 2013 to 2022; **table S4.** Differences of means of ragweed seed germination and viability rate in the burial experiment performed in Hungary; **table S5.** Climatic variables in the 10 years of the experiment at the burial sites Kaposvár and Groß-Enzersdorf.
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Review Article

Responses of invasive and native plant species to drought stress and elevated CO₂ concentrations: a meta-analysis

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Abstract

Superior trait responses of invasive plant species to their native counterparts determine invasion success under various environmental conditions. To date, numerous experimental studies have compared the physiological and growth trait responses of invasive plant species to native ones in simulated drought or CO₂ enrichment conditions; however, these studies have not recently been summarised. Here, we conducted a global meta-analysis using 48 experimental studies to determine whether there are generalisable differences between invasive and native plant species in terms of their physiological and growth trait responses to drought and elevated CO₂ and which traits potentially facilitate plant invasion in these conditions. The results indicate that the magnitude of responses do not differ substantially between invasives and natives for most traits under drought or elevated CO₂. Under drought stress, the photosynthetic rate, stomatal conductance, shoot biomass and total biomass decreased in both plant groups, supporting the contention that plants, irrespective of their origin, are negatively affected in water-limited environments. By contrast, we found that elevated CO₂ increased water-use efficiency, shoot biomass and total biomass and decreased stomatal conductance in both invasives and natives, indicating that both plant groups grow vigorously in such conditions. Compared with estimates for natives, invasives were taller and invested more biomass to roots under drought and showed greater allocation to shoot biomass under elevated CO₂. Although there were no substantial differences in the magnitude of responses in most studied traits, the differential growth responses in invasives may confer an advantage over natives under decreased water availability and high CO₂ concentrations.

Key words: Effect size, environmental variation, invasion ecology, non-native plants, quantitative synthesis, trait-based comparison

Introduction

Invasive plant species exert multifaceted effects on native biodiversity and many ecological processes (Culliney 2005; Vilà et al. 2011; Seebens et al. 2018; Smith et al. 2018; Pyšek et al. 2020). Therefore, it is of great importance to elucidate the mechanisms underlying plant invasions for the conservation of native biodiversity and restoration of native plant communities. Various plant traits, including physiological, growth and reproductive features, are thought to contribute to invasion processes (Drenovsky et al. 2012a; Divišek et al. 2018; Mathakutha et al. 2019) and these traits often show variation along environmental gradients in



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Copyright: © Norul Sobuj et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0) different ecosystems (Zheng et al. 2009; Milanović et al. 2020; El-Barougy et al. 2021). It has been suggested that, if invasive plant species exhibit superior trait responses in physiological and morphological attributes to those of resident native species in a plant community under altered environmental conditions, then they should outperform their native counterparts (Leffler et al. 2014; Mathakutha et al. 2019; Westerband et al. 2021) and this could explain how they become widespread and abundant in resident plant communities. To date, considerable experimental research has been put forth to investigate this notion by comparing the performances of numerous invasive species to their native competitors across taxa and biomes under simulated environmental conditions (Matzek 2012; Anderson and Cipollini 2013; Funk et al. 2016; He et al. 2018; Gufu et al. 2019; Henn et al. 2019), but the findings remain controversial. Therefore, a quantitative synthesis combining the results of independent studies is essential to draw a general conclusion about whether invasive plant species outperform native species across different environments.

Until recently, only a few studies compared the performance of invasive plant species and their native counterparts in various environmental conditions at a global scale (Daehler 2003; Sorte et al. 2013; Liu et al. 2017). In their meta-analysis, Sorte et al. (2013) showed that non-native invasive and native plant species respond similarly to various water regimes and temperatures and increased CO₂ concentrations benefitted more the former plant group, while Liu et al. (2017) revealed a significant difference in performance between these two plant groups under those environmental conditions. Both of these syntheses pooled values for various traits, including physiology, morphology and fitness-related traits, into a single metric in response to various environmental gradients. However, different traits do not respond in the same direction along environmental gradients (Leffler et al. 2013; Santamarina et al. 2022) due to trade-offs and resource constraints (Grime 2006; Ramírez-Valiente et al. 2010; Lau and Funk 2023). Additionally, some traits can be more responsive than others or do not respond at all to a particular environmental condition (Richards et al. 2006; Van Kleunen et al. 2010; Palacio-López and Gianoli 2011; Funk et al. 2016). Therefore, it is imperative to synthesise existing knowledge and bridge the gap in our understanding of how similar physiological and growth traits of invasive and native species respond to drought and elevated CO₂.

Given the importance of understanding invasive-native trait divergence in the context of environmental variation, a good number of studies have focused on differences in physiological and growth trait responses between these two plant groups in stressful conditions (e.g. Hwang and Lauenroth (2008); Funk et al. (2016); Huang et al. (2017); El-Barougy et al. (2020)). It has been posited that, under stressful environments, characterised by limited water, nutrients or light availability, both invasive and native plant species would downregulate their physiology and growth stimulation due to the unavailability of resources (Funk and Vitousek 2007; González et al. 2010; Santamarina et al. 2022). In drought stress, in particular, decreased water availability is predicted to constrain photosynthetic carbon assimilation because of decreased stomatal openness and other physiological adjustments in plants (DeFalco et al. 2003; Casper et al. 2006; Godoy et al. 2011; Drenovsky et al. 2012b). Accordingly, numerous studies have reported that, in water-limited environments, plants show decreases in the net photosynthetic rate and stomatal conductance, irrespective of species origin, thereby resulting in a

shift in biomass investment or overall reduction in plant growth (Drenovsky et al. 2012b; Larson and Funk 2016; Santamarina et al. 2022). However, comparative studies have also shown that invasive plant species often exhibit various attributes that confer some drought tolerance, such as thick leaves and greater allocation to below-ground structures in conditions where water is limited (Funk and Vitousek 2007; Grotkopp and Rejmánek 2007; Van Kleunen et al. 2010), but see Cavaleri and Sack (2010). Further, decreased water availability can be advantageous for invasive plant species over their native counterparts because the former could have a wide range of physiological niches and greater tolerance to environmental stresses (Funk and Vitousek 2007; Davidson et al. 2011; Funk 2013). Therefore, in some regions, particularly in arid and semi-arid systems, limited water availability may put many native plant species at a disadvantage, as demonstrated by Ding et al. (2021) and Drenovsky et al. (2012a, b). However, a general relationship between drought stress and the increased dominance of invasive plant species over native ones has yet to be established. Recently, some evidence also has suggested that drought stress has larger detrimental effects on growth, physiology and reproductive traits of some invasive species than the native ones within recipient communities (Valliere et al. 2019; Kelso et al. 2020; Liu et al. 2024).

The patterns in trait differences between invasive and native species may change with environmental conditions. It has widely been suggested that many invasive plant species will thrive in high-resource environments because invasive plant species, by virtue of their traits, tend to have fast resource use strategies and that attribute often becomes even faster in resource-rich non-native areas (Davis et al. 2000; Ordonez and Olff 2013; Sardans et al. 2017; El-Barougy et al. 2020). Therefore, environments with high CO₂ concentrations, which is a source of carbon fertilisation for plants, may favour invasive species over resident native species because the former have advantageous traits (e.g. high potential photosynthetic rate, high specific leaf area, high growth rate etc.) that facilitate rapid carbon assimilation and use (Vilà et al. 2007; Raizada et al. 2009; Anderson and Cipollini 2013). In agreement with this assumption, many earlier studies reported that atmospheric CO₂-enrichment benefits invasive species more by promoting their physiological functions, such as photosynthetic carbon assimilation and water-use efficiency and by accelerating their growth performance such as height and biomass accumulation than those of their native counterparts growing in common conditions (Vilà et al. 2007; Song et al. 2010; Lei et al. 2011; Blumenthal et al. 2013). However, several previous studies that support that elevated CO₂ concentrations would improve the growth and fitness of invasive species over those of natives, also suggest that many native species can profit equally as invasives in such conditions because the effect of elevated CO₂ concentration is more closely related to the species than to the origin of the species (Vilà et al. 2007; Song et al. 2010; Lei et al. 2011; Tooth and Leishman 2013). Therefore, a relevant question is whether co-existing invasive species would outperform natives, as many predict, under elevated CO₂ concentrations. Understanding whether invasive and native species have different or similar responses to elevated CO₂ concentrations would help predict future risks from invasions under such conditions.

In this meta-analysis including the past and most recent literature data, we aim to compare the responses of invasive and native plant species in terms of their physiological and growth traits to drought stress and elevated CO₂ concentrations. Although many different traits may determine the success of invasive

plant species, we focused on a subset of traits associated with physiology (i.e. net photosynthetic rate, stomatal conductance and water-use efficiency) and growth (i.e. height, specific leaf area, shoot biomass, root biomass, total biomass and root-shoot ratio). We included only these above-mentioned traits in the current synthesis because these physiological and growth traits were more frequently investigated in the retrieved studies. Given the substantial importance of plant attributes in shaping invasion success in resident communities, if a consistent difference in various physiological and growth traits between these two plant groups can be identified where invasive species exhibit significantly higher values for a suite of traits than the native ones, this may provide insight into which traits potentially can facilitate plant invasions in drought stress and in environments with high CO₂ concentrations in various ecosystems. We hypothesised that drought stress will constrain physiological and growth trait responses in both invasive and native plant species, but invasive species would still show greater tolerance to drought because of their higher plasticity and as they are more efficient at using limiting resources relative to native species adapted to such systems (Davidson et al. 2011; Heberling and Fridley 2013; Funk et al. 2016). We also hypothesised that an elevated CO₂ concentration would stimulate physiological and growth trait responses in both invasive and native plant species, as performance typically improves in resource-rich environments, irrespective of species origin; however, invasives will benefit more due to their ability to quickly take up available resources and their inherent faster growth than the native species (Tecco et al. 2010; Godoy et al. 2011).

Methods

Literature survey

A literature survey was conducted to obtain peer-reviewed articles and thesis papers that report the responses of invasive and native plant species to drought stress and/ or elevated CO₂ concentrations. For the purpose of the current study, a 'native' plant species was defined as a species that is not invasive in the study area or elsewhere and an 'invasive' plant species was defined as a species that was introduced and became invasive in the study area. To retrieve a large sample of studies, Science Direct, PQDT (ProQuest Dissertations and Theses) Global, Springer Archive, Wiley Online Library, Oxford University Press, JSTOR (Biological Science), Proquest (Natural Science Collection) and Google Scholar were used as literature sources. The following keywords or phrases were used in various combinations to search for relevant papers: "climate change or environmental change or environmental fluctuation or environmental stress or extreme climatic event or drought or drought stress or water stress or precipitation variability or elevated carbon dioxide or elevated CO₂ or carbon dioxide enrichment" AND "invasive or alien invasive or non-native invasive or non-native invasive" AND "native". Relevant literature was also retrieved by scrutinising the reference lists of papers identified by this search and from prior meta-analyses conducted on the topic (Sorte et al. 2013; Liu et al. 2017). In a few papers, it was not clear whether the introduced species was invasive or not from the description. In such cases, an additional search on the web was performed to confirm the status of those species. Our survey only included studies published in English.

Study inclusion and data extraction criteria

All retrieved studies were assessed individually. Studies were included in the meta-analysis if the following three criteria were met: 1) The study included at least one invasive and one native plant species in the same experiment. If a study involved several invasive and native plant species and they were not presented invasive-native pairwise, they were paired for the purpose of invasive-native comparison, based on their phylogenetic relatedness and/or shared growth forms. In some studies, several individual species were presented as an invasive or native plant group. Such groups were considered as an individual entity and included in the meta-analysis. All the experiments carried out in growth chambers, greenhouses, open top chambers (OTC), free air CO₂ enrichment (FACE) systems, mesocosms and field conditions were included; 2) The experimental plants were exposed to at least two treatment levels (i.e. control and treatment) for drought stress or elevated CO_2 ; 3) The study reported at least one of the following physiological or growth traits: net photosynthetic rate, stomatal conductance, water-use efficiency, specific leaf area, height, shoot biomass, root biomass, root-shoot ratio or total biomass of invasive and native plant species.

Mean values, sample sizes and variances (i.e. standard deviation or standard error) were extracted for the selected traits directly from texts or tables for all studies meeting the eligibility criteria. The web-based tool WebPlotDigitizer (Rohatgi 2014) was used for extracting the preceding information from studies in which they were shown in graphs or figures. In studies in which the measure of variance was expressed as standard error, it was converted to standard deviation to meet the effect size calculation criteria. Standard deviation was derived from standard error according to the following equation: standard deviation = standard error × \sqrt{n} , where n is the sample size. Data were extracted and compiled for a focal trait based on the following rules: 1) When experimental plants were grown in both monoculture and polyculture (competition), data were extracted only from monoculture because the targeted plants may perform differently under competition, influencing the results for focal treatments; 2) When a study had more than two levels for a focal treatment, data for the control and the highest level of manipulation relative to the control were extracted; 3) When experimental plants were subjected to other treatments alongside the focal treatments in a factorial design, data from the control condition were extracted only for the non-focal treatments; 4) When data were presented for multiple sampling dates, data were extracted only from the latest sampling date; 5) When a study reported ranges for the sample size, the lowest sample size within a range was obtained; 6) When data were presented in various measurement units for a specific trait across studies, they were converted to a common unit; 7) For photosynthetic rate, stomatal conductance or water-use efficiency, when a study presented data for any two of the three traits, values for the third trait were derived using the following equation: water-use efficiency = photosynthetic rate/stomatal conductance (Anderson and Cipollini 2013; He et al. 2018); 8) For total biomass, shoot biomass or root biomass, when a study presented data for any two of the three traits, values for the third trait were derived using the following equation: total biomass = shoot biomass + root biomass; 9) For shoot biomass, root biomass or root-shoot ratio, when a study presented data for any two of the three traits, values for the third trait were derived using the following equation: root-shoot ratio = root biomass/shoot biomass.

Effect size calculation and meta-analysis

The effect sizes were computed and meta-analyses were performed using the MetaWin statistical programme (Rosenberg et al. 2000a). In this synthesis, natural log response ratio (lnR) was used as the effect size metric which estimates proportionate changes between experimental and control groups (Hedges et al. 1999; Palacio-López and Gianoli 2011). The lnR was preferred over other measures of effect size calculation because the current meta-analysis included plant species of various growth forms (i.e. herbs, shrubs and trees) and the proportionate changes between experimental and control groups minimise the influence of plant size (Hedges et al. 1999; Palacio-López and Gianoli 2011). Firstly, lnR was computed for each specific trait in each invasive and native species in response to drought stress or elevated CO_{2} concentrations. The ln*R* was calculated using the following equation (Hedges et al. 1999): $\ln R = \ln(X_t) - \ln(X_c)$, where X_t and X_c represent the mean values of the samples measured in the treatment and control groups, respectively. The variances associated with $\ln R(V)$ were calculated as follows (Hedges et al. 1999): $V = S_{\perp}^2/N_{\perp}X_{\perp}^2$ + $S_c^2/N_c X_c^2$, where N_t and N_c denote the number of samples and S_t and S_c indicate the standard deviations in the treatment and control groups, respectively. In a few studies, neither the standard error nor standard deviation was reported. In such cases, the average value of X/S_1 or X/S_2 in other studies was calculated to derive a reference S_t or S_c value, respectively (Van Groenigen et al. 2011; Sun et al. 2020).

To understand the overall mean effect of drought stress or elevated CO₂ on a specific trait in invasive or native plant species, meta-analyses were conducted separately for each focal trait using the individual effect sizes and variances computed above. Mixed-effects models were preferred to perform the analyses with the assumption that there can be true random variation amongst the effect sizes originating from different studies (Rosenberg et al. 2000b; Borenstein et al. 2010). In our final models, species status (invasive or native) was used as a fixed factor and the selected studies included in the synthesis were used as a random factor to estimate the mean effect of drought stress or elevated CO₂ on the response variables (physiological and growth traits). The response of a specific trait to drought stress or an elevated CO₂ concentration was considered significant if the 95% confidence interval of the mean effect size estimate (calculated, based on 999 permutations) did not intersect with zero. A positive or negative mean effect size estimate indicates that the effect of drought stress or an elevated CO₂ concentration increased or decreased the response of a specific trait, respectively. Whether the response of a particular trait under drought stress or elevated CO₂ concentrations differs significantly between invasive and native plant species was also evaluated using the test statistic $Q_{\rm B}$ and associated *p*-values as a guide (Gurevitch and Hedges 2020). The $Q_{\rm B}$ statistic follows a chi-square (χ^2) distribution under the null hypothesis with m-1 degrees of freedom, where m is the number of subgroups in the meta-analysis (Rubio-Aparicio et al. 2017; Spineli and Pandis 2020). Publication bias was evaluated using funnel plots, which are widely used in meta-analyses, with a symmetrical distribution of scattered points around the zero-effect size indicating the absence of bias (Light and Pillemer 1986; Borenstein et al. 2021). The funnel plots were generated by plotting the effect sizes calculated, based on $\ln R$ on the x-axis and precision (1/SE) on the y-axis (Suppl. material 1: figs S1, S2). A visual inspection of the funnel plot showed minimal evidence of asymmetry.

Results

Based on the study selection criteria, we identified 48 empirical studies published from 1991 to 2022 (Suppl. material 1: table S1). The majority of the studies were conducted in North America and Asia, notably in the United States (31%) and China (27%), whereas studies from Europe (10%), South America (9%) and Africa (4%) were under-represented. Studies were mainly carried out in the greenhouses (58%), followed by mesocosms (15%), growth chambers (13%) and field conditions (6%) (Suppl. material 1: table S1). Potting soil was preferred as a substrate material in the majority of the experiments (67%), while field soil was used in the remaining studies (33%) (Suppl. material 1: table S1). Across studies, 74 invasive and 94 native plant species were exposed to either drought stress or elevated CO₂ concentration and most of the plants were herbaceous (86%). Our full dataset comprised 308 pairs of cases for comparisons between invasive and native plant species (drought stress: 157; elevated CO₂ concentration: 151) distributed across different physiological and growth traits (Figs 1, 2). We found that the most common plant attribute used to compare the performance between invasive and native plant species was total biomass in both drought stress (22%) and CO₂ enrichment (30%) treatments. Other common attributes that were studied were the root-shoot-ratio, specific leaf area and shoot biomass under drought stress and photosynthetic rate, stomatal conductance and specific leaf area under elevated CO₂ concentrations.

Our meta-analyses indicate that drought stress had negative effects on physiological functions and growth performance in both invasive and native plant species (Fig. 1). Compared with estimates in control individuals, plants subjected to water constraint exhibited a significantly lower photosynthetic rate (invasive: lnR = -0.32, CI = -0.55 to -0.09; native: lnR = -0.40, CI = -0.61 to -0.19), stomatal conductance (invasive: lnR = -1.73, CI = -2.46 to -0.99; native: lnR = -2.14, CI = -2.65 to -1.64), shoot biomass (invasive: lnR = -0.48, CI = -0.65 to -0.31; native: lnR = -0.68, CI = -0.86 to -0.51) and total biomass (invasive: lnR = -0.60, CI = -0.72 to -0.48; native: lnR = -0.60, CI = -0.72 to -0.48), regardless of their species origin (Fig. 1). In response to drought stress, a significant reduction was also detected in height growth (lnR = -0.25, CI = -0.35 to -0.15) and root biomass production (lnR = -0.36, CI = -0.46 to -0.25), but only in native plant species (Fig. 1). In native plant species, however, decreased water availability led to a significant increase in the root-shoot ratio (lnR = 0.25, CI = 0.13-0.36) (Fig. 1). When the magnitudes of the performance responses were compared between invasive and native plant species under drought stress, we found significant differences in height growth ($Q_{\rm B}$ = 5.87, df = 1, p = 0.015) and root biomass accumulation (Q_{R} = 19.19, df = 1, p = 0.000); native species showed greater decreases in height and allocation of biomass to the root system than those in invasive counterparts (Fig. 1, Table 1). Under decreased water availability, we also detected a marginally significant difference in the root-shoot ratio ($Q_{\rm B}$ = 3.56, df = 1, p = 0.059) between these two groups of plant species (Fig. 1, Table 1). In other studied traits, no significant differences were detected between invasive and native plant species, although water-limited conditions tended to have greater negative effects on photosynthetic carbon assimilation, stomatal conductance and shoot biomass production in native species than invasives (Fig. 1, Table 1).



Figure 1. Mean effect sizes of drought stress on the physiological and growth trait responses of invasive and native plant species. Error bars represent 95% confidence intervals around the mean effect sizes. The numbers in parentheses indicate the number of studies included in the meta-analyses and the number of individual effect sizes used to calculate the mean effect sizes, respectively. Asterisks denote a statistically significant difference between invasive and native plant species and *ms* indicates a marginally significant difference.

Table 1. Results of a meta-analysis of the effect of drought stress on the physiological and growth trait responses of invasive and native plant species. $Q_{\rm B}$ statistics and associated *p*-values for the difference in the magnitude of the response between invasive and native plant species subjected to drought stress are presented.

Trait	Species group	Effect sizes			$Q_{\rm B}$ tests		
		Mean	Lower 95% CI	Upper 95% CI	$Q_{\scriptscriptstyle m B}$	df	P
Photosynthetic rate	Invasive	-0.32	-0.55	-0.09	0.28	1	0.599
	Native	-0.40	-0.61	-0.19			
Stomatal conductance	Invasive	-1.73	-2.45	-0.99	0.84	1	0.359
	Native	-2.14	-2.65	-1.64			
Specific leaf area	Invasive	0.05	-0.03	0.14	1.92	1	0.166
	Native	-0.03	-0.12	0.05			
Height	Invasive	-0.07	-0.17	0.04	5.87	1	0.015
	Native	-0.25	-0.35	-0.15			
Shoot biomass	Invasive	-0.48	-0.65	-0.31	2.69	1	0.101
	Native	-0.68	-0.86	-0.51			
Root biomass	Invasive	-0.03	-0.13	0.07	19.19	1	0.000
	Native	-0.36	-0.46	-0.25			
Root-shoot ratio	Invasive	0.09	-0.02	0.20	3.56	1	0.059
	Native	0.25	0.13	0.36			
Total biomass	Invasive	-0.60	-0.72	-0.48	0.00	1	0.988
	Native	-0.60	-0.72	-0.48			

An elevated CO₂ concentration promoted changes in the physiological and growth traits in both invasive and native plant species, irrespective of their origin (Fig. 2). In both invasive and native plant species, the water-use efficiency (invasive: lnR = 0.23, CI = 0.08–0.39; native lnR = 0.19, CI = 0.03–0.34), shoot biomass (invasive: lnR = 0.52, CI = 0.45-0.59; native: lnR = 0.31, CI = 0.22-0.41) and total biomass (invasive: lnR= 0.28, CI = 0.19–0.36; native: lnR = 0.18, CI = 0.10–0.27) were significantly higher under CO₂ enrichment than in ambient conditions (Fig. 2). Further, a high CO₂ concentration significantly increased the photosynthetic rate (lnR = 0.15, CI = 0.04–0.26), height (lnR = 0.15, CI = 0.06–0.25) and root biomass production (lnR = 0.27, CI = 0.11–0.43) in invasive species (Fig. 2). However, our meta-analyses indicated that stomatal conductance (invasive: lnR = -0.20, CI = -0.35 to -0.05; native: lnR = -0.38, CI = -0.53 to -0.22) declined significantly in both invasive and native plant species subjected to elevated CO₂ concentrations (Fig. 2). While CO₂ enrichment benefitted both invasive and native plant species with respect to physiological and growth traits, there was a significant difference in shoot biomass accumulation ($Q_{\rm B} = 11.43$, df = 1, p = 0.001), based on species origin (Fig. 2, Table 2). In response to elevated CO₂ concentrations, invasive species exhibited significantly greater above-ground biomass production than that of their native counterparts. Moreover, we detected a marginally significant difference in photosynthetic carbon assimilation ($Q_{\rm B} = 2.86$, df = 1, p = 0.091) between these two plant groups grown in environments with high CO₂ concentrations (Fig. 2, Table 2). However, the magnitude of responses of most studied traits did not differ significantly between invasive and native plant species, although we observed a trend towards more positive effects of elevated CO₂ concentrations on invasive species (Fig. 2, Table 2).



Figure 2. Mean effect sizes of elevated CO_2 concentrations on the physiological and growth trait responses of invasive and native plant species. Error bars represent 95% confidence intervals around the mean effect sizes. The numbers in parentheses indicate the number of studies included in the meta-analyses and the number of individual effect sizes used to calculate the mean effect sizes, respectively. Asterisks denote a statistically significant difference between invasive and native plant species and *ms* indicates a marginally significant difference.

Trait	c	Effect sizes		$Q_{\rm B}$ tests			
	Species group	Mean	Lower 95% CI	Upper 95% CI	$Q_{\scriptscriptstyle m B}$	df	Þ
Photosynthetic rate	Invasive	0.15	0.04	0.26	2.86	1	0.091
	Native	0.02	-0.09	0.13			
Stomatal conductance	Invasive	-0.20	-0.35	-0.05	2.69	1	0.101
	Native	-0.38	-0.53	-0.22			
Water use efficiency	Invasive	0.23	0.08	0.39	0.19	1	0.660
	Native	0.19	0.03	0.34			
Specific leaf area	Invasive	-0.04	-0.11	0.03	0.06	1	0.813
	Native	-0.03	-0.10	0.04			
Height	Invasive	0.15	0.06	0.25	0.24	1	0.622
	Native	0.12	0.02	0.21			
Shoot biomass	Invasive	0.52	0.45	0.59	11.43	1	0.001
	Native	0.31	0.22	0.41			
Root biomass	Invasive	0.27	0.11	0.43	2.24	1	0.135
	Native	0.12	-0.01	0.24			
Root-shoot ratio	Invasive	0.10	-0.01	0.21	0.20	1	0.653
	Native	0.16	-0.07	0.40			
Total biomass	Invasive	0.28	0.19	0.36	2.33	1	0.127
	Native	0.18	0.10	0.27			

Table 2. Results of a meta-analysis of the effect of elevated CO_2 concentrations on the physiological and growth trait responses of invasive and native plant species. Q_B statistics and associated *p*-values for the difference in the magnitude of response between invasive and native plant species subjected to elevated CO_2 concentrations are presented.

Discussion

Responses of invasive and native plant species to drought stress

We predicted that both invasive and native plant species would show decreased performance in response to drought stress. Our meta-analysis indicated that the photosynthetic rate, stomatal conductance, shoot biomass and total biomass decline significantly in both groups of plants growing under reduced water availability. These results are consistent with numerous earlier studies showing that, when invasive and native plant species of various growth forms are subjected to simulated drought stress, they decrease their physiological functions, including photosynthetic carbon assimilation and stomatal conductance (Garcia-Serrano et al. 2009; Molina-Montenegro et al. 2011; Ding et al. 2021) and growth performance, including biomass production (Nernberg and Dale 1997; Huang et al. 2017; Valliere et al. 2019; Santamarina et al. 2022), in different ecosystems in arid, temperate and Mediterranean regions. In response to drought stress, plants often partially or completely close their stomata to limit water loss; this physiological response results in a decrease in net photosynthetic rates (Drenovsky et al. 2012b; Hatfield and Dold 2019; Ding et al. 2021). Over the long term, reduced photosynthetic rates in plants limit carbon gain and subsequently this results in decreased biomass production. Our findings, in combination with others, support the contention that both invasive and native plant species, irrespective of their origin, would suffer in water-limited environments because of downregulation of physiological processes and decreases in growth performance (Domenech and Vila 2008; Drenovsky et al. 2012b; Pan et al. 2017; Valliere et al. 2019).

We further hypothesised that the negative effects of drought stress will be greater for natives than for invasive species. Our results showed that, while invasive and native plant species were similar with respect to most traits under drought conditions, natives had significantly lower height growth and root biomass production when compared to their invasive counterparts. This indicates that invasive species were taller and invested more biomass into below-ground growth than did native species in environments with decreased water availability. For invasive species, modifications in biomass allocation could be beneficial under drought conditions. Increased allocation to the root system could be a key adaptation mechanism for the invasive species in decreased water availability, because roots can strongly affect water and nutrient acquisition in plants (Lopez-Iglesias et al. 2014; Larson and Funk 2016), especially in arid and semi-arid systems (Drenovsky et al. 2012b; Ding et al. 2021). As compared to the native species, greater allocation to roots by invasive species has previously been reported in a few large-scale experimental studies involving water constraint. For example, in a community-level comparison with 12 pairs of invasive and native plant species in California, Grotkopp and Rejmanek (2007) revealed that invasive species allocated more biomass to root tissue than did their co-occurring native species. Therefore, greater biomass allocation to the root system can have significant implications for the growth and survival of invasive species and, consequently, to the community dynamics and species distributions in water-limited conditions.

Contrary to our expectation, significant performance differences between invasive and native plant species existed only in height growth and root biomass production amongst the eight physiological and growth traits we analysed in response to drought stress. Therefore, strong evidence for invasive plant species differing substantially from native species in water-limited environments was lacking. We argue that, if the variation exists in only two traits, as was found in this global meta-analysis in height and root biomass between invasive and native plant species, it is unlikely that invasives will outcompete natives based solely as a result of these response differences in drought conditions. Our findings are consistent with those of earlier studies (e.g. Funk et al. (2016); El-Barougy et al. (2020); Westerband et al. (2021)) and support the argument that trait differences between invasives and natives should be weak or even absent under stressful conditions. Recently, Lau and Funk (2023) suggested that, in stressful conditions where resources are in constraint, invasive and native plant species compete for the same pool of limiting resources; therefore, these two groups of species should not be so different in terms of their performance since the maladaptation to extreme conditions prevents them from becoming established. Accordingly, a broad-scale comparison of performance differences between invasive and native plant species across contrasting climatic conditions including decreased water availability revealed that environmental filtering has led to similar trait values in native and invasive herbaceous plants (Tecco et al. 2010). Likewise, El-Barougy et al. (2020) also revealed that invasive and native plant species have overlapping trait values for specific leaf area, height and above-ground biomass under limited soil water or nutrient availability. These findings provide a support to the long-held notion that invasive and native plant species should be similar in most traits because of strong abiotic filters in stressful environments.

Responses of invasive and native plant species to CO₂ enrichment

Fitting with our expectation, CO₂ enrichment promoted physiological processes and growth changes in both invasive and native plant species. We found that, irrespective of species origin, plants grown in environments with a high CO₂ concentration had considerably higher water-use efficiency, shoot biomass and total biomass. Our results also demonstrate that stomatal conductance decreased significantly in both species groups subjected to elevated CO₂ concentrations. Reduced stomatal conductance may have contributed to the increased instantaneous water-use efficiency under elevated CO₂ concentrations, as observed previously in many species, including invasives and natives grown at high CO₂ concentrations (Ainsworth and Long 2005; Guerrieri et al. 2019; Hatfield and Dold 2019; Mathias and Thomas 2021). Moreover, in response to CO₂ enrichment, there were significant increases in the photosynthetic rate, height and root biomass, but only in invasive plant species. The increased photosynthetic rate in invasive species could explain their increased growth under an elevated CO₂ concentration as compared with that of plants grown in ambient conditions (Song et al. 2010; Lei et al. 2011; Blumenthal et al. 2013). Overall, our results are largely consistent with many earlier studies showing that raising atmospheric CO₂ concentration generally improves physiological functions and increases biomass accumulation in plants regardless of their origin in many ecosystems (Anderson and Cipollini 2013; Hager et al. 2016; He et al. 2018), although the effect can vary, based on the plant growth form and competitive ability of species (Sasek and Strain 1991; Vilà et al. 2007; Gufu et al. 2019).

Many earlier studies underlined the success of invasive species as a function of invasive-native performance differences across various conditions, such as environments with high CO₂ concentrations, high soil nutrients or ample water availability (e.g. Vilà et al. (2007); Funk et al. (2016); Sardans et al. (2017); Valliere et al. (2019); Musso et al. (2021)). Accordingly, in this study, we predicted that invasive plant species would respond more strongly than native species to elevated CO₂ concentrations because they generally possess growth and allocation traits that allow rapid carbon capture. Our comparison between the responses of invasive and native plant species to CO₂ enrichment showed that the only significant difference was in shoot biomass. The greater increment in above-ground biomass production under an elevated CO₂ concentration in invasive species than in their native competitors may enhance their competitive abilities and this could potentially be a mechanism promoting the success of invasion with ongoing increases in CO₂ concentration. Although some earlier studies have yielded contradictory results (e.g. Bradford et al. (2007); He et al. (2018)), our findings are in line with several other studies which also assessed a higher allocation of biomass to above-ground tissues in invasives species of different growth forms over their native counterparts at high CO₂ concentrations in various experimental conditions. As an example, Hager et al. (2016) found that an elevated CO₂ concentration stimulated shoot biomass in invasive grasses much more than that of their native competitors in a greenhouse experiment, even though the two species groups had similar responses in specific leaf area and conductance. Similarly, a FACE study also reported a greater increase in above-ground biomass production in an invasive forb as compared to a dominant native grass in response to CO₂ enrichment (Blumenthal et al. 2013).

Our findings are surprising considering that invasive plant species are presumably more successful than their native counterparts on a global scale and in high resource environments. Numerous empirical studies previously found a divergence in

responses between invasive and native plant species to elevated CO₂ concentrations in a long suite of traits including photosynthetic rate, stomatal conductance, specific leaf area, height, root biomass, total biomass and reproduction (Smith et al. 2000; Huxman and Smith 2001; Hättenschwiler and Körner 2003; Baruch and Jackson 2005; Song et al. 2009; Manea and Leishman 2011; Ibrahim et al. 2021) and these physiological and morphological traits play a significant role in invasion success in novel environments. While we detected a significant difference between native and invasive species in shoot biomass and a marginally significant difference in the photosynthetic rate, other traits did not vary with species origin. Here, a significant performance difference between these two species groups in only one of the nine assessed traits lends only weak support to our hypothesis. It has been reported that responses of plant traits to elevated CO, concentrations are species-specific and vary significantly amongst invasive species and amongst native species (Poorter 1993; Vilà et al. 2007; Rogers et al. 2008; Song et al. 2009; Sullivan et al. 2010; Lei et al. 2011; Tooth and Leishman 2013). Therefore, in this study, the difference between invasive and native species might not have been detected in most traits because of high interspecific variability within each species group. These results suggest that many invasive species are unlikely to dominate in environments with high CO₂ concentrations; however, both invasive and native species are likely to grow vigorously.

Study constraints

Our findings do not provide a clear indication whether invasive plant species will dominate over natives in drought stress or elevated CO₂ concentrations. We acknowledge some limitations associated with this synthesis. First, most of the plant species included in this study were herbaceous (86%) (Suppl. material 1: table S1). Previously, Tecco et al. (2010) and Westerband et al. (2021) compared trait differences between invasive and native plant species across a broad range of species and climatic conditions in several ecosystems and suggested that herbaceous invasive and native species growing in the same conditions do not exhibit trait differences. However, this was not the case for woody invasive species, in which functional attributes differed from those of woody natives in all ecosystems and conditions (Tecco et al. 2010). Therefore, marginal trait differences between invasive and native species in this meta-analysis could be driven by the dominance of herbaceous species. Here, we did not attempt to analyse our data separately based on plant life forms owing to the small sample sizes. Additional empirical studies that compare the physiological and morphological trait differences between woody invasive and native plant species growing in drought stress or CO₂ enrichment conditions could lead to more precise predictions.

Moreover, the majority of the studies included in this meta-analysis were short-term, mostly lasting one growing season (90%) (Suppl. material 1: table S1). However, responses of plant species to drought stress or CO_2 concentration gradients could vary over long time periods, especially at different life stages in biannual and perennial plants (Leadley et al. 1999; Stöcklin and Körner 1999; Laube et al. 2015); hence, the differences in plant attributes between invasive and native species can become more apparent over time. For example, long-term analyses have suggested that invasive species are far better able to respond to altered environmental conditions by adjusting their phenology than those of native plant species and this property facilitates invasion at the community level (Willis et al. 2010). Further, in this synthesis, we only retrieved data from monocultures since

only a few studies examined experimental plants grown in competition. However, in introduced communities, invasive plant species commonly occur together with their native counterparts and grow in competition with each other. Thus, our results should be interpreted with caution when considering the real world, because we only evaluated the main effects of drought stress and elevated CO_2 and excluded the interacting effects of competition and a focal treatment (see Vila et al. 2021 for interaction effects).

Finally, we did not evaluate various traits, such as reproduction, survivorship, allelopathy and susceptibility to herbivory, owing to limited data availability and these traits may differ between invasive and native plant species in environments with decreased water availability or high CO_2 concentrations (Horton and Clark 2001; Anderson and Cipollini 2013; Duell et al. 2021) and may also play a role in invasion success in such altered conditions. Hence, including a broader spectrum of traits, beyond those related to physiological processes and growth, can be helpful for robust predictions of the relative success of invasives and natives under drought and elevated CO_2 concentrations.

Conclusions

The findings of this meta-analysis indicate that drought stress suppresses the performance of both invasive and native plant species, while an elevated CO₂ concentration stimulates physiological processes, except stomatal conductance and growth traits in both plant groups. Our results also indicate that, compared with native plant species, invasive species had a significantly greater height growth and invested more biomass to below-ground root system in drought conditions and had a greater allocation to above-ground biomass production in elevated CO₂ concentrations. These differential responses of invasive plant species in growth traits may provide them with an advantage in adaptation over native species under decreased water availability and CO₂ enrichment. However, the magnitude of responses in most traits did not differ substantially between invasive and native plant species, indicating that invasive species are unlikely to outcompete natives in these altered environmental conditions. This certainly does not mean that invasive species will not be pervasive in the future, as they are capable of greater allocation to below-ground and above-ground biomass production compared with that of native counterparts under variable environments. Still, we need to be careful when predicting a bleak future regarding intensive dominance of invasive plants across different environmental conditions.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

NS and CB conceptualised and designed the study; NS extracted and analysed the data and developed graphs and tables; NS wrote the first draft of the original manuscript; KS and CB reviewed and edited the manuscript. All authors approved the submission to the journal.

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Data availability

Datasets were shared in the open access file directories of Figshare https://doi.org/10.6084/m9. figshare.27924888.

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Supplementary material 1

Additional information

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Data type: docx

- Explanation note: **table S1.** Overview of the experimental studies included in the meta-analysis. **fig. S1.** Funnel plot representing the relationship between effect size (lnR) and the inverse of the standard error (SE) in drought stress. **fig. S2.** Funnel plot representing the relationship between effect size (lnR) and the inverse of the standard error (SE) in elevated CO2 concentration.
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