RESEARCH ARTICLE



Flowers of *Impatiens glandulifera* as hubs for both pollinators and pathogens

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Abstract

Flower infestation by pathogens may influence pollination effectiveness. At the same time, by sharing infested flowers, pollinators increase transmission of pathogens. In the presented study we identified fungi that colonised flowers of the invasive alien Himalayan balsam Impatiens glandulifera, one of the most nectar rewarding plants in Europe, as well as its pollinators. We determined factors (e.g., plant size, length of flower lower sepal and the width of its entry, air temperature and sun illuminance) that affect pathogen species presence and pollinators numbers. The study was conducted in three regions in Poland differing in time from the I. glandulifera invasion onset. It allowed embedding our results in the context of the evolution of increased competitive ability (EICA) hypothesis. With reference to this hypothesis we tested whether I. glandulifera from the two younger populations are more frequently pollinated than individuals from the old one, which may be a result of the higher infection prevalence in the flowers of individuals from the latter population. Harmful primary pathogens of I. glandulifera (e.g., Botrytis cinerea and Fusarium graminearum) were identified from its flowers. Although the knowledge of the impact of the recorded pathogen species on the pollinators that transmit them is still limited, these pathogens are known to cause devastating diseases of native plant species and to incur significant economic losses in crops. Therefore, the facilitation of their transmission by *I. glandulifera* in the invaded communities may pose a serious threat both to native biodiversity and nearby crop production. We did not find support for the EICA hypothesis that flower release from pathogens may increase the pollinator's activity. Bombus hortorum was the most frequent visitor in the youngest surveyed population, while B. pascuorum was most frequent in the two others. So far the dominance of B. hortorum as a pollinator of I. glandulifera has not been recorded. A possible explanation is that flowers in the youngest population, with significantly wider entries than in the two older ones, were more accessible for this large bumblebee. We suggest that the shifts in flower dimensions may result from the evolutionary processes and/or phenotypic plasticity;

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however, this suggestion needs to be confirmed in further studies. At the same time, it can be expected that exceptionally frequent visits of *B. hortorum* in flowers of *I. glandulifera* in the youngest population may contribute to increasing transmission rate of pathogen species to the new native host plants that are particularly associated with this pollinator.

Keywords

Bumblebee activity, EICA hypothesis, floral traits, Himalayan balsam, intraspecific and interspecific transmission, pathogen hotspot, post-introduction shifts, pathogen spillback

Introduction

Himalayan balsam *Impatiens glandulifera* is native to the western Himalayas, while as an alien species it is known from Europe (35 countries), North America (Canada, United States, Mexico), Oceania (New Zealand) and Asia (Japan) (CABI 2022). The negative impact of *I. glandulifera* on native biodiversity and human economy has been widely demonstrated. This invasive species was reported to compete successfully with co-occurring plants, reducing their diversity by 25% (Hulme and Bremner 2005); moreover, its negative impact on animals occurring in invaded habitats was also documented (Ruckli et al. 2013). *I. glandulifera* may decrease pollination of wild growing plants (Chittka and Schürkens 2001) as well as crops (Najberek et al. 2021), whose yield often depends on the availability of pollinators (Klein et al. 2007). At the same time, high costs of the species eradication, reaching £10/m² (Tanner et al. 2008), make its control complicated or even impossible to achieve in areas where *I. glandulifera* is widespread.

I. glandulifera is characterized by traits that facilitate its invasion success. It was shown that it has a high photosynthetic capacity and growth rate (Ugoletti et al. 2011). Impressive antimicrobial and antioxidant properties of the species were also discovered (Szewczyk et al. 2016) as well as its high dispersal abilities along watercourses: seeds of this balsam can float and persist viable for over 2 months in still water (Najberek et al. 2020a). *I. glandulifera* is an annual species which spreads only via seeds and its autonomous selfing abilities are relatively low (Vervoort et al. 2011; Najberek et al. 2022a), therefore, high attractiveness for pollinators is crucial. The species attracts pollinating insects by producing the extraordinary volumes of nectar, amounting to 0.3 mg hourly per flower (Chittka and Schürkens 2001). Therefore, in this study we investigated pollinators of *I. glandulifera* and factors that may influence their activity, with the main focus on pathogens that occur in flowers of the balsam species.

Flowers are part of the phyllosphere which contains all aerial organs of plants. In general, in contrast to the rhizosphere and endo-rhizosphere, the phyllosphere microbiome was poorly studied to date (Timmusk et al. 2020). In the presented study, we isolated fungal endophytes that colonised *I. glandulifera* flowers, identified them molecularly and assessed their harmfulness and economic impact for plants and crops. It is known that the shared use of flowers leads to pathogen transmission between plants and their pollinators and that floral traits may influence disease transmission (McArt et al. 2014). Although the first study on this phenomenon was published already in 1994 (Durrer and Schmid-Hempel 1994), only 11 experimental studies concerning flowers as hotspots aiding plant and/or pollinator pathogens dispersal have been reported since then (Shykoff and Kaltz 1997; Goodell 2003; Ruiz-González and Brown 2006; Ruiz-González et al. 2012; Fouks and Lattorff 2013; Cisarovsky and Schmid-Hempel 2014; Graystock et al. 2015; Adler et al. 2018; Bodden et al. 2019; Figueroa et al. 2019; Yousefi and Fouks 2019). In the presented study we focused on the two floral traits: the length of the flower's lower sepal and the width of its entry. These traits determine both the accessibility of the host plant for particular groups of pollinators (Inouye 1980) and likelihood of pathogen transmission (Bodden et al. 2019). Flower shape is associated with the length of pollinator visit, which in turn influences the likelihood of the defecation inside the flower and consequently – the risk of pathogen transmission via faeces (Bodden et al. 2019). Notably, longer visits increase also likelihood of transmission of pathogens attached to the surface of the pollinator's body (Cisarovsky and Schmid-Hempel 2014). It is also important that it takes longer for infected pollinators to learn floral cues than it does for healthy ones; also perception of these cues during a visit is longer for infected pollinators, which extends visiting time and consequently - may also increase the likelihood of pathogen transmission (Figueroa et al. 2019). The flower structures play also a role in protecting or exposing pathogens to environmental hazards, which infuences the likelihood of pathogen survival (Cisarovsky and Schmid-Hempel 2014). Longer nectar tubes, for example, protect pathogens better than shorter ones (Durrer and Schmid-Hempel 1994). In turn, secreting of unique chemical compounds (secondary metabolites), as is the case with *I. glandulifera* flowers (Vanderplanck et al. 2019), may decrease pathogen survival. On the other hand, the bell-shaped flowers in this species are more susceptible to infections than flat-formed flowers of other species (Graystock et al. 2015). It should also be noted that the flowering potential may be affected through infections that occur before the flowering phase. For example, Shykoff and Kaltz (1997) found that the initial flowering of diseased male plant is retarded, however, the infection results also in increased number of produced flowers. This in turn allows plants to conceal their infections from pollinators (Shykoff and Bucheli 1995).

In the presented study we selected three populations of *I. glandulifera* that significantly differed in age. The population age, determined on the basis of literature data documenting invasion of the species in Poland (Tokarska-Guzik 2005; Zając et al. 2011), was important in the context of the evolution of increased competitive ability (EICA) hypothesis (Blossey and Notzold 1995). The hypothesis assumes that the enemy pressure on newly introduced populations of alien species is low, hence individuals may reduce their investment into defence against enemies and re-allocate the saved resources into the improvement of competitive abilities. We assumed that *I. glandulifera* may re-allocate the saved resources into traits that determine its high attractiveness for pollinators. We tested whether *I. glandulifera* individuals from the young and middle aged population are more frequently pollinated than individuals from the old population. Moreover, these populations were tested for fungal pathogens in the flowers visited by pollinators in order to indicate the population with the highest infections prevalence. Basing on the EICA assumptions, we hypothesised that young and middle aged populations of the species should be less infected than the old one. Importantly, such dependence may drive higher pollination success in the two former populations. The flower pathogens aspect has never been tested using invasive alien plants from populations varying in age.

In the presented study, pollinators and flower pathogens of the one of the most nectar rewarding plants in Europe, invasive alien *I. glandulifera* (Chittka and Schürkens 2001), were investigated. We tested also floral traits that determine pollinator visits and may influence pathogen transmission. In addition, the study was conducted using populations of different ages, which allowed to embed the collected dataset in the context of the EICA hypothesis assumptions. Moreover, based on our results as well as the reliable literture, we determined whether infected flowers of widely distributed *I. glandulifera* may pose a serious threat to the native plants and crops as well as to pollinators that also could become victims of pathogens that they are carrying between flowers.

Methods

Study area

The experiment was conducted in 2020 in southern Poland. Individuals of *I. glandulifera* were surveyed in three study regions – the Izerian Foothills, Kraków and Muszyna – differing in terms of the species introduction year (Table 1). The oldest Polish population of *I. glandulifera*, dating back to 1890s, occurs in the Izerian Foothills (Tokarska-Guzik 2005). From this source population the species dispersed to adjacent regions. In Kraków the species appeared in 1980s (Table 1), and it was probably the source of its further eastward expansion. In Muszyna *I. glandulifera* has been noted since 2010s (Zając et al. 2011). In each of the three regions the plants were surveyed along three rivers (Table 1). One survey per each locality was conducted, between 9 and 18 September (Table 1).

Plant measurements and weather conditions

At each locality 10 neighbouring individuals of *I. glandulifera* growing at the path margin were randomly selected and marked with a unique ID. Stem height and diameter of individuals were measured to include their size (Suppl. material 2: table S1) in the analyses; moreover, the number of flowers and seed capsules of individuals were also counted to assess their flowering power (see *Statistical analysis*). The hue of the flowers (pinkish or reddish) was also assessed.

The surveys were conducted on warm and rainless days. During each survey data loggers (i-Button DS1921G) were used to measure air temperature at 10-minute intervals, while hand-held environmental metre (Extech 45170CM) measured sun illuminance (lux) and wind speed (m/s) at 20-minute intervals.

Table 1. Characteristics of localities of the invasive alien *I. glandulifera* differing in population age in three study regions (Izerian Foothills, Kraków and Muszyna) in southern Poland; the surveyed plants always occurred at riversides.

Region	Year of introduction	Locality with coordinates	River name	Survey date
	(references)	(latitude, longitude)		
Izerian Foothills	1890s (Tokarska-	Zgorzelec (51.103805, 14.982722)	Nysa Łużycka	9/17/2020
	Guzik 2005)	Leśna (51.021625, 15.269387)	Bruśnik	9/17/2020
		Mirsk (50.973962, 15.365690)	Czarny Potok	9/18/2020
Kraków and	1980s	Kraków (50.038255, 19.897358)	Wisła	9/10/2020
surrounding areas	(Zając et al. 2011)	Szczyglice (50.086645, 19.814899)	Rudawa	9/10/2020
		Zabierzów (50.097423, 19.806040)	Rudawa	9/9/2020
Muszyna and	2010s	Andrzejówka (49.342193, 20.819197)	Poprad	9/14/2020
surrounding areas	(Zając et al. 2011)	Żegiestów (49.374850, 20.785117)	Poprad	9/14/2020
		Milik (49.347806, 20.851281)	Milik	9/15/2020

Pollinators counting

Over a 60-minute period during each survey the number of pollinators visiting flowers of the selected 10 plants was counted. The counting was always conducted in the morning (at 9:30 h) or in the afternoon (at 13:30 h). Pollinators were defined as all insects visiting flowers to collect pollen or nectar that may carry pollen from male (anther) to female (stigma) flower organs. Following the methodology adapted for *I. glandulifera* and *I. parviflora* in our previous study (Najberek et al. 2021), flights of the pollinators were tracked, and IDs of subsequently visited plants were noted; the pollinators were not captured but identified at flowers and their size was roughly assessed by assigning to one of three categories (small, medium, large).

Flower measurements

At the end of each survey 10 flowers (or less, depending on availability) of each plant individual were cut off and their profiles were photographed against a millimetre paper background (Canon EOS 60D, Canon EF 100 mm f/2.8 Macro USM lens and ring flashlight). The images were used to assess: the length of lower sepal and the width of flower entry. Subsequently, digital images were analysed with ImageJ software (ver. 1.51 k), and the area of one flower side was evaluated; this corresponds to 1/2 of the total flower area (Najberek et al. 2021). The data on length, width and area of flowers are included in Suppl. material 2: table S1.

Detection and molecular identification of fungi

The cut off flowers were inserted in paper bags (one bag per one plant) and transported in a portable freezer. Molecular identification of fungal pathogen species was carried out in the laboratory of Department of Systematic and Environmental Botany of Adam Mickiewicz University in Poznań (Poland). The flowers were surface-sterilized in 75% ethanol (30 s), 4.5% sodium hypochlorite (3 min 30 s), 75% ethanol (15 s), and then rinsed in sterile water. After sterilization, the flowers were cut into fragments and placed on Petri dishes with PDA medium and chloramphenicol (one flower per dish). To confirm the efficiency of the sterilisation process, 50 µl of rinse water was spread onto potato dextrose agar (PDA) and incubated at room temperature for 14 days. The addition of an antibiotic to the medium was used to exclude bacteria from the analysis, thus only pathogenic fungi were obtained from the plant tissues. In total, 585 flowers of 90 plant individuals were checked for the fungal presence. The dishes were placed in an incubator at 25 °C. They were observed every day, and emerging fungi were successively transplanted to new plates. For the identification of fungi, the fungal isolates were grouped into morphotypes based on macroscopic characteristics, such as the appearance and colour of the mycelium. Then, isolates representative of each morphotype were analysed using molecular methods.

The DNA was isolated using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol and was stored at -20 °C. A pair of primers, ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), was used to amplify the ribosomal cassette, which consisted of partial SSU, ITS1, 5.8S, ITS2 and partial LSU rDNA. The PCR reaction was conducted in a 25-µl volume containing 2.5 µl of 10X buffer, 2.5 µl of 2.5 mM dNTP mix, 0.5 µl of each primer at 10 µM, 0.5 µl of DNA Taq polymerase, 13.5 µl of nuclease-free water, and 5 µl of DNA template. Amplification was conducted in a thermocycler using a programme with the following parameters: 2 min at 95 °C; 37 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C; and finally 5 min at 72 °C. The PCR products were purified using alkaline phosphatase and exonuclease I and directly cycle-sequenced with ABI BigDye Terminator ver. 3.1 (Applied Biosystems, USA). The obtained sequences were edited with Chromas (www.technelysium.com.au) software and were compared to those published in the European Molecular Biology Laboratory (EMBL) nucleotide databases and in the NCBI (www.ncbi.nlm.nih.gov) databases using BLAST (Altschul et al. 1990). A positive identification of a species was confirmed if ≥98% of the ITS region sequence identity was shared with the reference sequence from the databases. The obtained sequences were submitted to the GenBank.

Statistical analysis

The data were analysed with generalised linear models (GLMs) using SPSS ver. 26.0 (IBM Corp. 2016); all used data is presented as Suppl. material 1. The models with the lowest Akaike information criterion (AIC) were chosen (Burnham and Anderson 2002). Three GLM models were created: (1) for all recorded pollinators, (2) for bumblebees solely and (3) for pathogens identified from the flowers (Suppl. material 2: table S2). All models assumed the Poisson distribution for the numerical data. Pairwise contrasts were applied for between-group comparisons with adjustment for multiple tests using least significant difference (significance level 0.05).

In the first model the number of all recorded pollinators, calculated per plant individual per survey ('N pollinator records'), was a target variable (sample size = 216). In the base model (Suppl. material 2: table S2), the following fixed effects were included: pollinator identified to a species or a higher taxonomic group ('Pollinator'; single records of *Bombus lapidarius* and *Psithyrus vestalis* were excluded from the analysis), study region representing population age of the surveyed plant individuals ('Region'), reproduction potential of the plant ('Flowering power'), area of flower profile ('Flower area'), hue of flower ('Flower hue'), height of the plant ('Stem height'), weather variables ('Temperature', 'Sun radiation' and 'Wind speed'), area of *I. glandulifera* patches at particular localities ('Cover area') and morning / afternoon survey time ('Day time'). Two interactions were also included: 'Region * Flowering power' and 'Region * Stem height' (Suppl. material 2: table S2). The 'Flowering power' was calculated from principal component analysis (PCA), based on two variables: (1) number of flowers and (2) number of seed capsules. In PCA, the Kaiser-Meyer-Olkin measure of sampling adequacy was 0.50, with a p-value in Bartlett's test < 0.001. The percentages of variance accounting for the two obtained components were 80.3% and 19.7%; the first component with an eigenvalue of 1.606 explained most of the variance.

The second GLM model was created only for bumblebee records as a target variable; as in the previous model, single records of *B. lapidarius* and *P. vestalis* were excluded from the analysis. This group of pollinators was most numerous. Data on bumblebees were also more precise than on other pollinators, thanks to the assessment of their size. Size assessment is particularly important in analyses of bumblebee effects, because meaningful size polymorphism occurs among workers (Mares et al. 2005). The following fixed effects were taken into consideration: bumblebee species ('Bumblebee'') and its size ('Bumblebee size'), 'Flowering power', 'Flower area' and three weather variables ('Temperature', 'Sun radiation' and 'Wind speed').

In the last model the number of recorded pathogen species per each surveyed plant was used as a target variable. Moreover, the following variables were included in the model as fixed effects: the number of recorded pollinators ('N pollinator records'; single records of *B. lapidarius* and *P. vestalis* were excluded from the analysis), 'Region' differing in population age, two weather variables ('Temperature', 'Sun radiation'), 'Flowering power', three variables associated with flower dimensions ('Flower area', 'Length of lower sepal' and 'Width of flower entry') and a single variable representing size of the surveyed plants ('Plant size'). In addition, the interaction between flower-ing power and study region differing in age was included in the model ('Flowering power * Region'). 'Plant size' was obtained from principal component analysis (PCA), based on two variables: (1) stem height and (2) stem diameter. In PCA, the Kaiser-Meyer-Olkin measure of sampling adequacy was 0.50, with a p-value in Bartlett's test < 0.001. The percentages of variance accounting for the two obtained components were 82% and 18%; the first component with an eigenvalue of 1.640 explained most of the variance.

Data resources

Raw data used to perform statistical analyses are provided as a file with supporting information.

Results

In total, we recorded 1828 pollinator visits (Table 2) and 97.26% of them were identified at the species level, 1.04% at the family/superfamily level, while 1.7% belonged to the *Bombus lucorum* complex. Bumblebee species dominated regardless of the studied region differing in the age of surveyed plants; including a single record of *Psithyrus vestalis* we recorded 1625 of bumblebee visits (88.9% of all visits; Table 2). Most of them were recorded at plants surveyed in Muszyna and surrounding areas (N = 1014; 55.5%), where *B. hortorum* was the most numerous species (N = 988, 97.4%; Table 2). In turn, the Izerian foothills and Kraków, even if combined, had fewer records than Muszyna (N = 814; Table 2), with *B. pascuorum* as the most frequently noted species (N = 216 and 212, respectively; 52.7% and 52.5%, respectively; Table 2). Moreover, in the Izerian foothills the honeybee *Apis mellifera* was the second most abundant species (Table 2).

In the statistical analysis conducted for all recorded pollinators, the model with eight fixed effects and one interaction was selected (Suppl. material 2: table S2). During the model selection procedure, the following variables were excluded from the best-fit model: flower hue, wind speed, day time; also interaction between regions differing in age and flowering power was excluded (Suppl. material 2: table S2).

In the best-fit model, the three variables (pollinator species, flowering power and cover area) and the interaction between the regions differing in age and stem height played a significant role (Table 3; Suppl. material 2: table S2). The most frequently recorded species visiting *I. glandulifera* flowers was *B. hortorum* (at p < 0.001 in all comparisons; Fig. 1; all statistics are included in Suppl. material 2: table S3). The second important species was *B. pascuorum* (at p < 0.02 in 5 of 8 comparisons; Fig. 1; Suppl. material 2: table S3). We also found that the number of pollinators increased with the flowering power (Table 3). Moreover, in patches with larger cover area the higher pollinator activity was noticeable (Table 3). In turn, the interaction between the regions

Pollinator species		N records		TOTAL:
	Izerian Foothills	Kraków	Muszyna	
Bombus hortorum	27	29	988	1044
Bombus pascuorum	216	212	9	437
Apis mellifera	134	33	11	178
Bombus terrestris	20	82	0	102
Bombus lucorum complex	0	31	0	31
Syrphidae	3	5	6	14
Bombus sylvarum	9	0	0	9
Vespula vulgaris	0	6	0	6
Apoidea	0	5	0	5
Bombus lapidarius	1	0	0	1
Psithyrus vestalis	0	1	0	1
TOTAL:	410	404	1014	1828

Table 2. The number of pollinators recorded at flowers of of *I. glandulifera* differing in population age in the three study regions (Izerian Foothills, Kraków and Muszyna) in southern Poland.



Figure 1. The estimated mean number of pollinators (\pm confidence intervals) recorded at flowers of *I. glandulifera* in the three study regions of different population age in southern Poland (Izerian Foothills, Kraków and Muszyna); groups with the same letter above the T-bars are not significantly different at p < 0.05; single records of *Bombus lapidarius* and *Psithyrus vestalis* were excluded from the analysis (see Methods).

Table 3. GLM best-fit model for the number of recorded pollinators. The following variables were included in the model: pollinator species (Table 2), three study regions differing in population age (Izerian Foothills, Kraków and Muszyna), flowering power (N of flowers and N of seed capsules computed into a single variable), flower area, stem height, weather conditions (air temperature, sun radiation), cover area as well as interaction between the study region differing in age and stem height. The estimated means \pm SE for pollinator species and region are *Bombus hortorum*: 14.247 \pm 1.876, *B. pascuorum*: 7.638 \pm 1.170, *Apis mellifera*: 4.103 \pm 0.851, *B. terrestris*: 5.200 \pm 1.467, *B. lucorum* complex: 1.635 \pm 0.861, Syrphidae: 0.734 \pm 0.533, *B. sylvarum*: 2.502 \pm 2.297, *Vespula vulgaris*: 2.530 \pm 2.850, Apoidea: 2.250 \pm 2.733, Izerian Foothills: 2.460 \pm 0.731, Kraków: 2.904 \pm 0.744, Muszyna: 4.774 \pm 1.515.

Effect	F	df	р
Pollinator species	7.31	205	< 0.001
Region	0.45	199	0.6
Flowering power	8.30	198	0.004
Flower area	0.74	198	0.4
Stem height	0.29	198	0.6
Temperature	1.60	198	0.2
Sun radiation	2.83	198	0.094
Cover area	3.44	198	0.065
Region * Stem height	3.18	199	0.044

differing in age and stem height demonstrated a different role of plant height in various habitat conditions (Fig. 2, Table 3). In Muszyna (the young population) taller plants were visited significantly more often than shorter ones (Fig. 2, Table 3), whereas, in the two remaining regions (middle age and old populations) longer stems did not have any effect on the number of recorded pollinators (Fig. 2, Table 3). However, the result of the interaction was not associated with the age of *I. glandulifera* individuals in particular regions. In Muszyna, where the relationship was significant, plants co-occurred with equally tall and very dense bushes, which may determine this result. It should be stressed as well that the result for region variable, that also represents population age of the surveyed plants, was non-significant, thus it can be assumed that the obtained result was not consistent with the EICA hypothesis assumptions.

In the analysis carried out solely for bumblebees, the model with all seven fixed effects was selected (see base model in Suppl. material 2: table S2). Except for flower area, all included effects played a significant role in shaping the activity of bumblebees at *I. glandulifera* flowers (Table 4). The results for particular bumblebee species (Suppl. material 2: fig. S1, table S4) showed the same pattern as in the analysis for all pollinators (Fig. 1); there was only one exception – the numbers of visits of *B. pascuorum* and *B. sylvarum* did not differ between each other, while there was a difference in the



Figure 2. The scatter plot with fit lines for the relationship between the number of visiting pollinators and stem height of *I. glandulifera* individuals in the three study regions of different population age in southern Poland (Izerian Foothills, Kraków and Muszyna). The coefficients of determination are R² Linear: 0.011, Izerian Foothills: R² Linear 0.007, Kraków: R² Linear 0.002, Muszyna: R² Linear 0.065.

Table 4. GLM best-fit model for the number of recorded bumblebees. The bumblebee species (Suppl. material 2: fig. S1), their size, flowering power (combined N of flowers and N of seed capsules), flower area and weather conditions (air temperature, sun radiation and wind speed) were included in the model. The estimated means \pm SE for bumblebee species and size are *Bombus hortorum*: 4.082 \pm 0.522, *B. lucorum* complex: 0.525 \pm 0.215, *B. pascuorum*: 2.678 \pm 0.364, *B. sylvarum*: 1.096 \pm 0.813, *B. terrestris*: 1.805 \pm 0.433, small: 1.166 \pm 0.273, medium: 4.664 \pm 0.813, large: 0.790 \pm 0.216.

Effect	F	df	р
Bumblebee species	8.82	233	< 0.001
Pollinator size	58.79	231	< 0.001
Flowering power	18.45	230	< 0.001
Flower area	1.60	230	0.2
Temperature	12.90	230	< 0.001
Sun radiation	64.38	230	< 0.001
Wind speed	7.38	230	0.007

model for all pollinators. We also found that medium-sized bumblebees were recorded more frequently than small and large ones (contrasts respectively: Estimate = 3.50, SE = 0.66, t = 5.33, p = 2.290E-7; Estimate = 3.87, SE = 0.71, t = 5.48, p = 1.091E-7; Suppl. material 2: fig. S2); at the same time, there were no differences between the numbers of visiting small and large bumblebees in this respect (contrast: Estimate = 0.38, SE = 0.71, t = -5.48, p = 1.091E-7; Suppl. material 2: fig. S2). Flowering power also influenced the obtained results (Table 4) in that the number of bumblebees visiting flowers increased with the estimate of the number of produced flowers. In turn, the area of flowers did not play any significant role (Table 4). Weather conditions had an impact on the obtained results (Table 4; Suppl. material 2: fig. S3) as shown by the number of recorded bumblebees increasing with air temperature (AVG = 19.4 °C, MIN = 14 °C, MAX = 27.1 °C) and decreasing with both sun radiation (AVG = 11765lux, MIN = 2000 lux, MAX >= 20000 lux) and wind speed (AVG = 0.6 m/s, MIN = 0 m/s, MAX = 2.5 m/s).

There were 16 species of fungal pathogens (Table 5) in the sampled flowers; GenBank IDs of the recorded species are included in Suppl. material 2: table S5. Some of them, including *Botrytis cinerea* and *Fusarium graminearum*, are very harmful pathogens of native plants and crops (Dean et al. 2012). The flowers from the Izerian Foothills were colonised by four times less pathogen species than the flowers from the two other regions (Table 5). Species belonging to the *Fusarium* genus were most frequently recorded (N = 27; 34.2% of all records in the studied regions). *F. sporotrichioides* dominated in the Izerian Foothills, *F. boothii* was the most frequent species in Kraków, and *Alternaria alternata* – in Muszyna (Table 5). It should be also noted that plant flowers were colonised by 1, 2 or 3 pathogen species (N = 36, 40.0%; N = 15, 16.7%; N = 4, 4.4%; respectively). Moreover, flowers of 35 of all plant individuals (38.9%) were not colonised by any pathogen species.

In the analysis of pathogen species records, the model with two fixed effects – study region and flowering power – and interaction between these two variables was selected (Table 6; Suppl. material 2: table S2). In turn, the following explanatory variables were

Species		N of records		TOTAL:
	Izerian Foothills	Kraków	Muszyna	
Alternaria alternata	1	6	8	15
Epicoccum nigrum	1	7	5	13
Fusarium boothii	0	8	0	8
Fusarium sporotrichioides	3	5	0	8
Alternaria tenuissima	0	1	3	4
Boeremia exigua	0	0	4	4
Epicoccum tritici	1	2	1	4
Fusarium acuminatum	1	2	1	4
Botrytis cinerea	0	0	3	3
Cladosporium cladosporioides	0	1	2	3
Fusarium avenaceum	1	2	0	3
Fusarium equiseti	1	2	0	3
Mucor hiemalis	1	0	2	3
Nigrospora oryzae	0	1	1	2
Fusarium graminearum	0	1	0	1
Stagonosporopsis ligulicola	0	0	1	1
TOTAL:	10	38	31	79

Table 5. The number of fungal species records revealed in three study regions differing in population age (Izerian Foothills, Kraków and Muszyna).

Table 6. GLM best-fit model for the number of recorded pathogen species. The pathogen species (Table 5), three study regions (Izerian Foothills, Kraków and Muszyna), flowering power (combined N of flowers and N of seed capsules) and interaction between region and flower power were included in the model. The estimated means \pm SE for region are Izerian Foothills: 0.283 \pm 0.096, Kraków: 1.098 \pm 0.160, Muszyna: 1.120 \pm 0.177.

Effect	F	df	р
Region	3.03	86	0.054
Flowering power	6.80	85	0.01
Flowering power * Region	4.92	86	0.01

excluded during model selection procedure: the number of recorded pollinators, temperature, sun radiation, flower area, length of lower sepal, width of flower entry and plant size (Suppl. material 2: table S2).

The number of pathogen species in flowers of *I. glandulifera* from the Izerian Foothills (the old population) was lower than that in flowers from the two younger populations from Kraków or Muszyna (contrasts respectively: Estimate = -0.81, SE = 0.187, t = -4.358, p = 3.692E-5; Estimate = -0.84, SE = 0.20, t = -4.157, p = 7.737E-5; Fig. 3); at the same time, Kraków and Muszyna did not differ in this respect (contrast: Estimate = -0.02, SE = 0.239, t = -0.091, p = 0.9; Fig. 3); the result was contrary to our assumption constructed based on the EICA hypothesis expectations (we assumed that the level of infestation should be lower in the young and middle age populations than in the old one). Moreover, we revealed that in Kraków and in the Izerian Foothills the number of pathogen species decreased with plants flowering power (Fig. 4, Table 6); interestingly, the result for Muszyna was opposite (Fig. 4).



Figure 3. The estimated mean number of pathogen species (\pm confidence intervals) recorded from flowers of *I. glandulifera* in the three study regions of different population age in southern Poland (Izerian Foothills, Kraków and Muszyna); groups with the same letter above the T-bars are not significantly different at p < 0.05.



Figure 4. The relationship between the number of pathogen species and flowering power for different regions. Flowering power was calculated by PCA (combined N of flowers and N of seed capsules); the measurements were carried out in the three study regions of different population age in southern Poland (Izerian Foothills, Kraków and Muszyna).

Discussion

Infected plants may be less attractive to pollinators because pollinators can usually recognise contaminated flowers and thus they are able to avoid getting infected (Fouks and Lattorff 2011). For example, it was shown that bumblebees are able to detect the odour of a parasite Crithidia bombi (Fouks and Lattorff 2013). It was also demonstrated that larval honey bee parasite, the fungus Ascosphaera apis, decreases pollinator visits of several plant species (Yousefi and Fouks 2019). At the same time, Drosophila melanogaster may distinguish safe food source from food with toxic microbes (Stensmyr et al. 2012). However, plants try to compensate for pathogens presence, for instance by producing more flowers and/or more nectar (Shykoff and Bucheli 1995; McArt et al. 2014). In flowers of I. glandulifera, characterised by very high nectar content (Chittka and Schürkens 2001) and extended flowering, compensation may play an important role. If this is the case, pathogens occurring in frequently pollinated flowers of *I. glandulifera* may have a higher chance to be transmitted to other plants that share pollinators with this alien plant. It should be stressed that although it was not directly tested in this study, there is ample evidence that the shared use of flowers leads to horizontal pathogen transmission between plant individuals of the same generation. In the first experimental study on the transmission Durrer and Schmid-Hempel (1994) demonstrated that parasite C. bombi was horizontally transmitted through bumblebees between the inflorescences of Echium vulgare. The transmission of the same parasite between the flowers was confirmed also in other experiments (Ruiz-González and Brown 2006; Ruiz-González et al. 2012; Cisarovsky and Schmid-Hempel 2014). The mechanism was confirmed also for other pathogens and plants. For example, bumblebee parasites – Apicystis bombi, C. bombi and Nosema bombi, and honeybee parasites – N. apis and *N. ceranae*, can be rapidly transmitted between infected and healthy flowers of purple Campanula cochleariifolia (Graystock et al. 2015). In turn, Bodden et al. (2019) in their study of floral cues of eight plant species and bumblebee Bombus impatiens as a vector, provided evidence on pathogen transmission between the visited flowers.

The interspecific transmission of pathogens in *I. glandulifera* may determine the lower pollination rate of co-occurring native plants. The transmission probably occurs because native species have lower nectar content than *I. glandulifera* and it is probable that they are not able to mask the presence of pathogens so efficiently as this alien species. Its domination in terms of nectar production may also be manifested in neighbouring crops, whose yield success usually depends on the availability of pollinators (Klein et al. 2007). For example, it is likely that dangerous pathogens are interspecifically transmitted from flowers of *I. glandulifera* to flowers of tomato *Solanum lycopersicum* during alternating bumblebee visits to flowers of the two species (Najberek et al. in review). Such alternating visits were also noted between another alien balsam, *I. parviflora*, and co-occurring strawberries *Fragaria* ×*ananassa* (Najberek et al. 2021). Interestingly, in the presented study fungal pathogen *Alternaria tenuissima* (formerly *Alternaria tomato*) was identified in flowers of *I. glandulifera* (in Kraków and in Muszyna). This primary pathogen causes spot diseases of many crops, including nailhead spot of tomato and leaf spots of strawberries (Horst 2013a, Bagherabadi 2015). Thus,

it cannot be excluded that *I. glandulifera* could facilitate spreading of this harmful crop pathogen and consequently – decrease yields.

In Muszyna, the cosmopolitan pathogen Botrytis cinerea was also recorded from the sampled flowers of I. glandulifera. This pathogen causes grey mould disease in more than 200 crop species and could be transmitted by insects (Woodford et al. 2002; Williamson et al. 2007). Moreover, B. cinerea is included as the second most important species in the list of top 10 fungal pathogens in molecular plant pathology (Dean et al. 2012); the list was constructed based on the scientific and economic pathogen importance. Interestingly, it was revealed that in greenhouse cultivations the pathogen spread was successfully reduced through inoculation with other fungal species, e.g., Trichoderma harzianum, used as biocontrol agents, distributed among flowers by bumblebees and bees. For example, they were successfully applied in strawberry cultivations using *B. terrestris* (Mommaerts et al. 2011). Moreover, it was also revealed that spores of B. cinerea may have some nutritional value for pollinators: their consumption with food increases longevity of workers of honey bees A. mellifera (Parish et al. 2020). A similar effect was obtained for diets supplemented with four species of Cladosporium genus, including C. cladosporioides (Parish et al. 2020). Notably, C. cladosporioides and A. alternate (both recorded from I. glandulifera flowers) may also play a positive role in plant-pathogen interactions. These two fungi are considered as less harmful secondary plant pathogens that may deter occurrence of primary pathogens with an invariably negative impact on plants and their propagules. In some circumstances the presence of secondary pathogens may even benefit a plant by limiting its infestation by primary pathogens (Liggitt et al. 1997; Najberek et al. 2018). In the sampled flowers we also found six Fusarium species (F. acuminatum, F. avenaceum, F. boothii, F. equiseti, F. graminearum, F. sporotrichioides). Fusarium species, classified as most harmful plant pathogens worldwide (Timmusk et al. 2020), are primary pathogens that cause a devastating fungal disease – Fusarium head blight (FHB). Like B. cinerea, F. graminearum, a highly destructive pathogen of all cereal species, is included in the list of top 10 fungal pathogens in molecular plant pathology (Dean et al. 2012); in our study, this pathogen was recorded in Kraków. Thus, it is highly possible that I. glandulifera invasion promotes transmission of at least two extremely dangerous plant pathogens.

Another fungal species detected during the study was a secondary pathogen *Mucor hiemalis* (recorded in the Izerian Foothils and Muszyna). It is a harmless saprophytic species producing secondary metabolites that are toxic to *Thielaviopsis paradoxa* – a pathogen that causes inflorescence brown rot disease; however, the same metabolites could be harmful to insects that may transmit them (Ziedan et al. 2013). Therefore, transmission of *M. hiemalis* may play both a positive and negative role in the pollinatorplant interactions. In Muszyna we also recorded *Boeremia exigua* which causes leaf spots of ornamental plants (e.g., hollyhock and rose-mallow; described as *Phoma exigua* by Horst 2013a) and likewise attacks crops (e.g., sweet potato and oregano; Zimowska 2015; Colmán et al. 2020). In turn, in Kraków and Muszyna we found *Nigrospora oryzae*. This is another pathogen that causes numerous crop diseases, e.g., Ripe Fruit Rot of tomato and Nigrospora Cob Rot of corn (Horst 2013b). This pathogen was found also in guts of bees (Moubasher et al. 2017); therefore, its transmission between flowers seems evident. Notably, *C. cladosporioides* was also identified from the same bee samples, as well as from samples of beetles and weevils (Moubasher et al. 2017). In Muszyna *Stagonosporopsis ligulicola* was also found. However, the impact of this pathogen has been poorly investigated to date. It is known that the species may be associated with ray blight disease of Asteraceae (Vaghefi et al. 2012); the major host of *Stagonosporopsis ligulicola* var. *ligulicola* is an ornamental hybrid *Dendranthema xgrandiflorum* (EPPO 2021).

In the presented study the aspect on floral traits that may influence transmission of pathogens between plant flowers was also taken into consideration. Because flower shape could play a significant role in pathogens transmission (Bodden et al. 2019), we tested the effect of width of flower entry and length of lower sepal of *I. glandulifera* flowers. They have a deep and tight corolla, and we assumed that the probability of pathogen deposition should increase with the dimensions of flower entry and its sepal. However, this expectation was not supported by our results, which is in line with the results obtained by Adler and co-authors (2018). The number of records of pathogen species was not associated with the number of visiting pollinators either. However, it should be stressed that we did not count the number of colonies per each recorded pathogen species. Thus, the quantitative data on infestation with particular pathogens was not taken into consideration in the statistical analyses, while such data may also yield additional insights into the studied phenomena. In turn, flowering power had an important role in the assessment. We found that the number of pathogen species may either decrease or increase with the number of flowers and seed capsules, depending on the study region invaded by populations of different age. In general, the probability of pathogen detection should increase with flowering power, because plants should tend to conceal their real infection level from pollinators by increased production of flowers (Shykoff and Bucheli 1995). This trend was noted in the youngest of the studied populations, in Muszyna, whereas in the middle age and old populations, in Kraków and in the Izerian Foothills, the opposite results were obtained. A possible explanation could be that the lower level of attack from the enemies in Muszyna (including all sources of damage) allowed the plants to relocate the resources into masking fungal infestation by producing more flowers. However, the result was not in line with this explanation, because the plants in Muszyna were not particularly effective in enemy release abilities (plants from the Izerian Foothills were most effective in this respect). Therefore, it is more probable that these differences are associated with secondary metabolites (i.e., polyphenol rich substances) secreted in I. glandulifera flowers (Vanderplanck et al. 2019). These metabolites might reduce prevalence of some of the pathogen species. The plants in Kraków and in the Izerian Foothills might have evolved to secrete more metabolites in flowers than the plants in Muszyna. However, this is only a supposition which needs to be tested in the future. Further tests should also include broad assessments of the impact of those metabolites on particular pathogen species. This aspect has been poorly explored to date. Vanderplanck et al. (2019) indicated only a single pathogen susceptible to I. glandulifera metabolites (Apicystis bombi) and three other species that were resistant to those metabolites (C. bombi, Nosema bombi, N. ceranae). In our tests, none of these pathogen species was detected. Notably, flowers of almost 40% of the surveyed plant individuals were not colonised by any pathogen species, while in the remaining 60% we always found between 1 and 3 species. We did not sample plants/crops co-occurring with

I. glandulifera, therefore, it cannot be assessed if the detected levels of floral pathogens were high, medium or low. However, we did it in a parallel experiment testing the impact of *I. glandulifera* on pollination of cultivated tomatoes *S. lycopersicum* (Najberek et al. in review). We identified significantly more pathogen species and their colonies (including *B. cinerea*) from the flowers of the invasive alien species than from the crop. Therefore, it can be assumed that although flowers of *I. glandulifera* may decrease prevalence of some primary pathogens (e.g., *A. bombi*), at the same time they provide a shelter for many other primary pathogens that are not prone to its floral secondary metabolites. Notably, the risk of primary pathogens transmission from flowers of the species dominate in a given area. The extraordinary attractiveness of *I. glandulifera* for pollinators results in a large number of visits to its flowers (Chittka and Schürkens 2001; Najberek et al. 2021), which may in turn increase the overall pathogen transmission irrespective of its local pathogen loads.

In our experiment, bumblebees were the most frequently noted pollinator group. Two species dominated - Bombus pascuorum (in Kraków and Izerian Foothills) and B. hortorum (in Muszyna). The former species was recorded as the dominant pollinator of I. glandulifera also in other European studies (Chittka and Schürkens 2001; Nienhuis et al. 2009; Najberek et al. 2021), whereas domination of *B. hortorum* has not been recorded to date. Because the length of its tongue is larger than in B. pascuorum and it prefers deeper flower corollas, we assumed that its dominance in Muszvna could be associated with shifts in dimensions of *I. glandulifera* flowers in this region. However, the flowers from Muszyna were smallest and the length of their lower sepal did not differ in comparison with the two other populations. Moreover, we found no evidence that flower size had a significant influence on the results. Therefore, the dominance of B. hortorum cannot be explained by the occurrence of deeper flower corollas in the individuals in Muszyna. We found, however, that the width of flowers entry in this locality was larger than both in Kraków and in the Izerian Foothills, which could account for the frequent records of *B. hortorum*. This bumblebee species is meaningfully larger than *B. pascuorum*, therefore the narrower flower entry might be a barrier for this common bumblebee in two other surveyed regions. Such shifts in flower dimensions may appear with time after alien species introduction in new areas and may result from the evolutionary processes and/or phenotypic plasticity (Agrawal 2001; Colautti and Barrett 2013). We also found that in Muszyna I. glandulifera individuals co-occurred with equally tall and very dense bushes, and only in this population stem height positively influenced the frequency of pollination. Therefore, future monitoring of evolutionary processes of the young population of I. glandulifera in Muszyna, where habitat conditions are more competitive and where *B. hortorum* is, surprisingly, the dominant pollinator, is particularly recommended. It may yield insights into the mechanisms determining post-introduction shifts that alien species are manifesting in populations at the invasion forefront (Blossey and Notzold 1995; Gruntman et al. 2017). It should be also noted that the two bumblebee species, B. pascuorum and B. hortorum, pollinate different host plants. In Southern Poland, the former one prefers (in the descending order) Trifolium pratense, Salvia glutinosa, Galeopsis speciose, Galeopsis pubescens and Lamium album, while the latter one - T. pratense, Centaurea jacea, Vicia cracca,

Cirsium oleraceum and *Cirsium vulgare* (A. Kosior, unpublished data). Therefore, it should be expected that frequent visits of *B. hortorum* to *I. glandulifera* with wider flowers entry in Muszyna may contribute to increased transmission of their pathogens to new native host plants that are pollinated by this species and commonly occur in this region (Flora Polski 2023). As pathogens of these native species have never been studied in Muszyna, it cannot be unequivocally assessed whether *I. glandulifera* was indeed the primary source of the infection, or if it had occurred before its invasion started. However, even if the native plants had already been infected from other source(s), frequent visits of *B. hortorum* to *I. glandulifera* are likely to enhance pathogen spread (spillback mechanism; see Kelly et al. 2009; Roy et al. 2017; Najberek et al. 2022b).

Brain size of bumblebees increases with their body size and it was revealed that individuals with larger brains have superior cognitive abilities to smaller ones (Mares et al. 2005). Larger brains allow, for instance, a better visual resolution (Spaethe and Chittka 2003) and faster learning (Worden et al. 2005). On the other hand, the colony costs of production of larger workers are relatively high, moreover, they have lower survivorship than medium and smaller ones (Kerr et al. 2019). In our experiment we found that bumblebee individuals of medium size were dominant visitors of flowers of I. glandu*lifera*, while the numbers of large and small bumblebees were threefold lower. A similar trend was noted in the study on B. vosnesenskii (Kerr et al. 2019), where such intermediate-sized workers were assessed as the most beneficial for the colony. We also found that the number of the recorded bumblebees increased with air temperature and decreased with sun radiation and wind speed. The average air temperatures that we recorded were optimal for bumblebee flights (15-25 °C; Pawlikowski et al. 2020), while minimal and maximal temperatures only marginally exceeded this threshold. Under higher air temperatures (> 30°C), the number of visits would probably start to decrease. Notably, it was shown that the number of workers' revisits to probed flowers also increases with air temperature (Najberek et al. 2021); repeated visits of flowers decrease feeding efficiency and may have consequences for the colony economy, as well as for the pollination rate of host plants (Leonard et al. 2011; Najberek et al. 2021). In another study (Xu et al. 2021) it was shown that sun radiation increases the body temperature of insects, which may cause overheating. Therefore, decrease in bumblebee activity with sun radiation, found in our experiment, is well understood. Increasing wind speed that disturbs the bumblebee flight could also be a reason for lower pollinator activity. Thus, it should be expected that I. glandulifera individuals growing in shaded and sheltered localities under optimal temperatures (15-25 °C during flowering phase) are most frequently visited by bumblebees. In turn, increased pollination rate determines faster spread and higher invasiveness of the species. Interestingly, we also revealed that the number of pollinator records increased with the area of *I. glandulifera* patches as well as with the numbers of flowers and seed capsules (analysed as flowering power). The results showed that larger patches of this invasive alien plant receive more attention from pollinators than smaller ones, because of superior flowering power and greater nectar reward.

In the presented study the associations between the EICA hypothesis (Blossey and Notzold 1995), pollinator activity and level of fungal pathogens that colonised flowers in populations of different age were investigated. However, we found no confirmation

that population age plays a role. Individuals from the young population were not more frequently pollinated than individuals from the two older ones. Therefore, it cannot be concluded that individuals from the young population could reduce their investment into defence against enemies and subsequently re-allocate the saved resources into the improvement of floral traits that facilitate their pollination success; e.g., the investment in larger flower size (non-significant in the presented study). The assumptions of the EICA hypothesis did not hold true in the results obtained for pathogens either. In this case, the lower number of pathogen species was recorded in the oldest population (the Izerian Foothills), while the most harmful plant pathogen species -B. cinerea and F. graminearum – were detected in Kraków (the middle age population) and in Muszyna (the youngest population). Interestingly, the association between the EICA hypothesis and pollinator activity was found in a non-invasive alien I. balfourii population in Zagreb, Croatia (Najberek et al. 2020b). It was demonstrated that the leaf damage by enemies in this population was significantly lower than in four older populations (Croatian Istria, Italian Insubria and Torino, and French Le Rozier), and at the same time pollination rate was highest. In addition, fungal pathogen Aureobasidium pullulans was frequently recorded from leaves and seeds in this population (Najberek et al. 2020b). This pathogen produces antibacterial and antifungal substances, which could increase the resistance against primary pathogens that permanently decrease the condition of the host plant (Bozoudi and Tsaltas 2018). Thus, although the invasive I. glandulifera and non-invasive I. balfourii are closely related (Janssens et al. 2009) changes that they undergo after the introduction in Europe are far from being parallel. It should be also noted that the EICA hypothesis was previously tested using I. glandulifera (Gruntman et al. 2017), however, different assumptions were verified. The authors assumed that the saved resources could be reallocated into allelopathic abilities of the species. They demonstrated that the leaf damage recorded in younger populations was significantly lower than that in the older ones, however, the damage was not associated with the level of allelopathic compounds secreted by the surveyed plants. Thus, neither in the previous studies on I. glandulifera (Gruntman et al. 2017), nor in the presented ones, the assumptions associated with the EICA hypothesis were confirmed.

Conclusions

Pollinators facilitate the spread of primary pathogens with invariably negative impact on plants, as well as less harmful secondary plant pathogens (their presence may even benefit a plant) and saprotrophs. In the presented study we demonstrated that the invasive alien *Impatiens glandulifera* may play a significant role in this process. The nectar-rich flowers of this species are very attractive for common pollinators and should be considered as a hot spot in intraspecific and interspecific pathogens transmission in the invaded communities. We identified each of three types of pathogens from the species flowers: primary pathogens, secondary pathogens and also saprotrophs. The most dangerous plant pathogens were *Botrytis cinerea* and *Fusarium graminearum*. These two fungal pathogens cause devastating diseases of native plant species and hundreds of crop species worldwide and are included in the list of top 10 fungal pathogens in molecular plant pathology (Dean et

al. 2012). Therefore, facilitation of their transmission by *I. glandulifera* invasion decreases crop production and increases economic losses. Unfortunately, this aspect of harmful impact of alien plant species, as *I. glandulifera*, is neglected. There is only a single experiment on negative influence of invasive alien plant species on crops (Najberek et al. 2021), whereas biological invasions may have critical consequences for human economy. Although the European Union has tightened the law on the possibility of spread and new introductions of the worst alien species (*I. glandulifera* is included in the regulation; European Commission 2017), the level of control of the most invasive alien species is still insufficient.

The surveyed *I. glandulifera* flowers were pollinated mainly by two bumblebee species, *Bombus pascuorum* and *B. hortorum*. The former one is known from its association with this plant species, while the latter has never been recorded as its dominant pollinator. Its domination was recorded in the youngest surveyed population of *I. glandulifera*, in which the flowers had wider flower entries than in two other populations. *B. hortorum* is a large bumblebee and narrower flower entry may be a barrier for this species. We suppose that the revealed shifts in flower shape of *I. glandulifera* may have occurred after its introduction in Europe. Nevertheless, this supposition needs to be confirmed in further experiments testing a post-introduction shift of floral traits of *I. glandulifera*. It should also be noted that exceptionally frequent visits of *B. hortorum* to *I. glandulifera* may contribute to a significant increase in the transmission rate from *I. glandulifera* flowers to new native host plants that it also frequently pollinates.

It is noteworthy that the results of our study could not be explained based on the assumptions of the EICA hypothesis. We found that population age of plants surveyed in different regions was not associated with the numbers of recorded pollinators; moreover, the results of flowers' infestation by primary pathogens were even contrary to these assumptions. In addition, in the presented study the evidence of negative impact of recorded pathogen species on host plants (including crops) is provided, whereas still little is known on the impact of those pathogens on the pollinators that transmit them. Single studies demonstrated that the interactions between plant pathogens and pollinators could be beneficial (e.g., *B. cinerea* may have nutritional value for *Apis mellifera*; Parish et al. 2020). However, it cannot be excluded that for some other pollinators, such as hoverflies, the same pathogens could be detrimental. It is clear that flower visiting may increase the risk of acquiring parasitic infection by pollinators (Durrer and Schmid-Hempel 1994). Therefore, further studies on the impact of pathogens on pollinator that are carrying them are strongly recommended.

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Supplementary material I

Raw data used to perform statistical analyses

Authors: Kamil Najberek, Wojciech Solarz, Wojciech Wysoczański, Ewa Węgrzyn, Paweł Olejniczak

Data type: Tables

- Explanation note: Raw data contains datasets on recorded pollinators and floral pathogens used in statistical analyses.
- 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.87.102576.suppl1

Supplementary material 2

Data on *I. glandulifera* individuals, bumblebees pollinating them, and additional information on statistical analyses

Authors: Kamil Najberek, Wojciech Solarz, Wojciech Wysoczański, Ewa Węgrzyn, Paweł Olejniczak

Data type: Tables, plots

- Explanation note: Supporting tables contain data on the size of the surveyed *I. glandulifera* individuals as well as on the statistical models selection. Supporting figures demonstrate data on bumblebee species recorded from *Impatiens glandulifera* as well as the influence of weather conditions on the obtained results.
- 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



Comparative mitogenomics of native European and alien Ponto-Caspian amphipods

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Abstract

European inland surface waters are home to a rich diversity of native amphipod crustaceans, many of which face threats from invasive Ponto-Caspian counterparts. In this study, we analyse mitochondrial genomes to deduce phylogenetic relationships and compare gene order and nucleotide composition between representative native European and invasive Ponto-Caspian taxa across five families, ten genera and 20 species (with 13 newly sequenced herein). We observe various gene rearrangement patterns in the phylogenetically diverse native species pool. *Pallaseopsis quadrispinosa* and *Synurella ambulans* exhibit notable deviations from the typical organisation, featuring extensive translocations of tRNAs and the nad1 gene, as well as a tRNA-F polarity switch in the latter. The monophyletic invasive Ponto-Caspian gammarids display a conserved gene order, primarily differing from native species by a tRNA-E and tRNA-R translocation, which reinforces previous findings. However, *Chaetogammarus warpachowskyi* shows extensive rearrangement with translocations of six tRNAs. The invasive corophiid, *Chelicorophium curvispinum*, maintains a highly conserved gene order despite its distant phylogenetic position. We also discover that native species have a significantly higher GC and lower AT content compared to invasive species. The mitogenomic differences observed between native and invasive amphipods warrant further investigation and could provide insights into the mechanisms underlying invasion success.

Keywords

invasive, mitochondria, native, nucleotide composition, Ponto-Caspian, phylogeny

Introduction

The European continent harbours a vast diversity of inland amphipod crustaceans, found in surface or subterranean, fresh or brackish waters (Barnard and Barnard 1983; Väinölä et al. 2008; Borko et al. 2021; Copilaș-Ciocianu and Sidorov 2022). Moreover, this diversity is significantly underestimated due to the widespread prevalence of cryptic species (Copilaș-Ciocianu et al. 2018; Eme et al. 2018; Wattier et al. 2020; Bystřický et al. 2022). However, a substantial proportion of this fauna faces threats from invasive species, climate change, eutrophication and other anthropogenic factors (Fišer et al. 2010; Rewicz et al. 2014; Maximov 2021; Arbačiauskas et al. 2022). One of the main challenges native amphipods encounter is competition and potential extinction due to the spread of invasive counterparts, particularly those originating from the Ponto-Caspian Basin (Rewicz et al. 2014; Arbačiauskas et al. 2017; Copilaș-Ciocianu et al. 2023).

The Ponto-Caspian region encompasses the Azov, Black, Caspian and Aral seas, as well as the lower stretches of their tributaries (Copilaș-Ciocianu et al. 2023a). This area is characterised by a unique endemic fauna, particularly adapted to wide salinity fluctuations (Reid and Orlova 2002; Paiva et al. 2018). Due to their environmental tolerance, many Ponto-Caspian endemics have become invasive, expanding their range beyond native borders, mainly colonising European inland waters and even reaching other continents (Bij de Vaate et al. 2002; Copilaș-Ciocianu et al. 2023a). Amphipods represent one of the most successful groups, with up to 40% of the species pool spreading outside their native range during the last century, mainly due to increased shipping activity, construction of canals and intentional introductions (Arbačiauskas et al. 2011; Copilaș-Ciocianu et al. 2023a). Invasive Ponto-Caspian amphipods can be competitively superior to the native species they encounter along invasion routes, leading to the native species decline and eventual extinction (Dermott et al. 1998; Grabowski et al. 2007, 2009; Bacela-Spychalska and van der Velde 2013; Šidagytė and Arbačiauskas 2016; Minchin et al. 2019).

Comparative studies involving both native and invasive species are essential for understanding invasion success. However, the underlying molecular and genetic mechanisms behind the success of Ponto-Caspian species invading new areas are not well-known and research is still in its early stages (Adrian-Kalchhauser et al. 2020; Mamos et al. 2021). The mitochondrial genome is a good candidate for comparative molecular studies, as mitochondria are crucial for the functioning of multicellular life and complete mitochondrial genomes are relatively inexpensive and easy to sequence due to recent advances in high-throughput sequencing and bioinformatic pipelines (Trevisan et al. 2019; Macher et al. 2020). Given that studies generally reveal strong differentiation in respiratory function amongst native and invasive aquatic species (Lenz et al. 2011; Lagos et al. 2017a, 2017b; Hraoui et al. 2020), it is reasonable to assume that the structure of the mitochondrial genome may provide insights into invasion success.

To date, relatively few mitochondrial genomes are available for invasive Ponto-Caspian amphipods and native European species, many of which were obtained from transcriptomic data and are, thus, of varying reliability (Krebes and Bastrop 2012; Macher et al. 2017; Cormier et al. 2018; Bojko 2020; Mamos et al. 2021). Of the 13 widespread invasive Ponto-Caspian species, the mitochondrial genomic structure is reliably known for four species from two genera (Dikerogammarus bispinosus, D. haemobaphes, D. villosus and Pontogammarus robustoides), while the mitogenome of Obesogammarus crassus is only known from transcriptomic reads, resulting in some regions having low coverage and potentially reduced reliability (Mamos et al. 2021). With respect to native European species, the situation is more severe, as reliable mitogenomes are available for only four species out of several dozen (and possibly hundreds of species): Gammarus duebeni, G. fossarum, G. lacustris and G. roeselii. (Krebes and Bastrop 2012; Macher et al. 2017; Cormier et al. 2018; Sun et al. 2020), while four more species have mitogenomes assembled from transcriptomic reads (G. pulex, G. wautieri, Echinogammarus berilloni and Pectenogammarus veneris) (Cogne et al. 2019; Mamos et al. 2021). For the purpose of this study, we treat G. roeselii as a native south-east European species, although we acknowledge its non-native status in central-western Europe (Csapó et al. 2020).

In this study, we compare the mitochondrial gene order, nucleotide composition and assess the phylogenetic relationships of native European and invasive Ponto-Caspian amphipods. We present a significantly expanded dataset that includes mitochondrial genomes representing most major native and invasive species in Europe. We present the first mitochondrial genomes of native *Synurella ambulans*, *Pallaseopsis quadrispinosa*, *G. jazdzewskii* and *G. varsoviensis*, the first DNA-based mitogenome for *G. pulex* and the first mitogenome of *G. lacustris* from Europe (previously sequenced only from the Tibetan Plateau (Sun et al. 2020)). Regarding the invasive species, we present the first mitogenomes for *Chaetogammarus ischnus* and *C. warpachowskyi*, the first DNA-based mitogenome for *O. crassus* and additional mitogenomes for *D. haemobaphes*, *D. villosus* and *P. robustoides*. Lastly, we present the first mitogenome for *Chelicorophium curvispinum*, the most widespread Ponto-Caspian corophiid amphipod. Our study provides new insights into the mitochondrial genomes of native European and invasive Ponto-Caspian amphipods, enhancing our understanding of their phylogenetic relationships and potentially uncovering key factors contributing to the invasion success of these ecologically important species.

Materials and methods

Sampling, laboratory protocols and sequencing

Animals used in the analyses were collected from Lithuania, Poland and Latvia between 2018 and 2020 using kick-sampling with a hand net (see Suppl. material 1 for detailed locality information). Specimens were stored in 96% ethanol in the field. Afterwards, the ethanol was exchanged several times and the material was stored at -20 °C. Specimens were identified using relevant keys (Eggers and Martens 2001; Copilaş-Ciocianu et al. 2014; Copilaş-Ciocianu and Sidorov 2022). The taxonomy of the focal taxa follows the most recent updates (Hou and Sket 2016; Sket and Hou 2018; Copilaş-Ciocianu and Sidorov 2022; Garcia-Paris et al. 2023; Horton et al. 2023).

We dissected the dorsal half of the animal (from head to urosome) using microsurgical scissors and fine needles to avoid contamination from the gut and extracted genomic DNA using the Quick-DNA Miniprep Plus Kit (Zymo Research) with the lysis step prolonged overnight. All specimens selected for high-throughput sequencing were also DNA-barcoded using the protocols described in Copilaş-Ciocianu et al. (2022) to further confirm morphological identifications.

After DNA extraction, we assessed quantity and fragment length of the genomic DNA using a FragmentAnalyzer (Agilent, USA). To fragment the DNA, the Covaris M220 system (Covaris, UK) was used targeting a fragment size of 250 base pairs. The fragmented DNA was then checked again on the FragmentAnalyzer system to confirm the quantity and length of fragments. The NEBNext Ultra II DNA Library Prep Kit and corresponding NEBNext Multiplex Oligos for Illumina were used to prepare shotgun genomic libraries following the manufacturer's protocol. The final library concentration and fragment size were confirmed on a TapeStation (Agilent) before manual equimolar pooling of samples. A negative control was processed together with the samples. It did not show any DNA when measured on FragmentAnalyzer and TapeStation before sequencing and was, therefore, not sequenced. The final library was sequenced using the Illumina NovaSeq 6000 platform with 2 × 150 bp read length at Macrogen Europe.

Bioinformatics, mitochondrial genome assembly and annotation

Raw data were checked for low-quality samples using the FastQC software and Illumina adapters were trimmed using Trimmomatic (Bolger et al. 2014). Strict quality filtering was applied to trimmed reads using vsearch, with reads truncated at the first base with a phred score < 15. Reads shorter than 100 bp were excluded from subsequent analysis. Per sample, ten million quality-checked reads were assembled using Megahit (Li et al. 2015) on the Naturalis high-performance cluster, with kmer lengths ranging from 15 to 115. The resulting contigs were imported into Geneious Prime (v.2022.2) and BLAST searches were conducted against a manually compiled reference library of amphipod mitochondrial genes (Macher et al. 2017; Mamos et al. 2021) downloaded from NCBI GenBank (https://www. ncbi.nlm.nih.gov/genbank/). Contigs were identified as potential mitochondria, based on BLAST results and contig lengths (between 10,000 and 20,000 bp). Potential mitochondrial contigs were subsequently annotated using Mitos2 (Donath et al. 2019). Annotations were manually checked and refined in Geneious Prime and gene sequences (nucleotide and protein) were extracted for subsequent phylogenetic analyses.

Nucleotide composition

We added the 13 mitogenomes obtained in this study to seven mitogenomes from previous studies, totalling 20 species, of which eight were Ponto-Caspian invaders and 12 native species (Table 1). Nucleotide composition was calculated for the entire mitogenomes using MEGA 6 (Tamura et al. 2013). To visualise patterns of composition amongst species, the percentage matrix of each of the four nucleotides was subjected to a Principal Component Analysis (PCA) using a variance-covariance matrix. A Permutational Multivariate Analysis of Variance (PERMANOVA) test with 9,999 permutations was used to detect differences in nucleotide composition between the native and invasive species groups. Furthermore, GC and AT content were separately compared between native and invasive species using a Mann-Whitney test. All analyses were conducted with PAST 4.10 (Hammer et al. 2001).

Family	Species	NCBI	Status	Mitogenome	A %	T %	G %	C %	Source
		number		length (bp)					
Corophiidae	Chelicorophium	CC6	Invasive	14867	37.8	30.6	12.6	19.1	This study
	curvispinum ¹								
Gammaridae	Chaetogammarus warpachowskyi ¹	CW4	Invasive	17336	35.2	35.9	10.9	18.0	This study
Gammaridae	Chaetogammarus ischnus ¹	EI4	Invasive	14694	32.5	33.9	12.1	21.5	This study
Gammaridae	Dikerogammarus bispinosus	OK173840	Invasive	15336	33.9	36.6	11.1	18.3	Mamos et al. (2021)
Gammaridae	Dikerogammarus haemobaphes ¹	DH3	Invasive	15258	31.9	34.2	13.1	20.9	This study*
Gammaridae	Dikerogammarus villosus ¹	DV4	Invasive	15176	32.7	35.1	12.3	19.9	This study*
Pontogammaridae	Obesogammarus crassus ¹	OC4	Invasive	15838	33.6	37.5	11.3	17.6	This study†
Pontogammaridae	Pontogammarus robustoides ¹	PR4	Invasive	15917	33.3	36.2	11.8	18.7	This study*
Crangonyctidae	Synurella ambulans	SA1	Native	15652	32.2	30.8	13.3	23.6	This study
Gammaridae	Gammarus duebeni	JN704067	Native	15651	32.5	22.0	31.5	14.0	Krebes and Bastrop (2012)
Gammaridae	Gammarus fossarum	KY197961	Native	15989	32.0	22.0	33.2	12.9	Macher et al. (2017)
Gammaridae	Gammarus lacustris	GL1	Native	18195	31.1	32.8	13.3	22.8	This study*
Gammaridae	Gammarus jazdzewskii	GZ1	Native	16136	34.6	34.4	11.4	19.5	This study
Gammaridae	Gammarus pulex	GP2	Native	14886	33.1	34.0	12.2	20.7	This study†
Gammaridae	Gammarus roeselii	MG779536	Native/	16073	33.9	32.9	12.3	20.9	Cormier et al.
			Non-native						(2018)
Gammaridae	Gammarus varsoviensis	GV1	Native	15482	31.1	32.8	13.2	22.8	This study
Gammaridae	Gammarus wautieri	BK059229	Native	13927	32.4	22.2	34.2	11.2	Cogne et al.
									(2019), Mamos et al. (2021)†
Gammaridae	Echinogammarus berilloni	BK059223	Native	14454	30.2	26.9	28.0	14.9	Cogne et al. (2019), Mamos
									et al. (2021)†
Gammaridae	Pectenogammarus veneris	BK059233	Native	14369	34.1	22.2	31.4	12.4	Cogne et al.
	-								(2019), Mamos
Pallaseidae	Pallacentesis anadrictimora	PO1	Native	16147	30.9	30.9	15.2	22.0	et al. (2021)†
1 andSCIUdC	1 unuscopsis quuarispinosa	1,71	inative	1014/	50.9	50.9	19.9	22.9	This study

Table 1. Overview of the species used in the comparative analyses.

¹ – Ponto-Caspian species; * – species whose mitogenomes were also sequenced in previous studies; † – species whose mitogenomes were previously assembled from RNA sequences.

Phylogenetic analyses

The purpose of these analyses was to place the focal taxa within the broader phylogenetic context of Amphipoda. In total, the data obtained in this study were combined with an additional 62 mitogenomes from literature, representing 25 families and 59 species, including one isopod outgroup, *Ligia oceanica* (see Suppl. material 1 for further details). The analyses were based on the 13 protein-coding genes and excluded the large (16S rRNA) and small (12S rRNA) ribosomal subunits. Protein-coding genes evolve in a more predictable manner than the erratic ribosomal units and can be confidently aligned. Each of the 13 genes was aligned separately by codon using MUSCLE (Edgar 2004) implemented in MEGA 6 with default options. All nucleotide alignments were protein translated using the invertebrate mitochondrial genetic code (translation table 5). Individual gene alignments were concatenated using SequenceMatrix (Vaidya et al. 2011). Both nucleotide and translated protein sequences were used in the phylogenetic analyses. The concatenated nucleotide matrix had a total length of 11,047 bp, while the protein matrix was 3,682 amino acids long. The best partitioning schemes (by codon) and evolutionary models for the nucleotide data were selected with PartitionFinder 2 (Lanfear et al. 2016).

Phylogenetic analyses were conducted within a Bayesian (BI) framework with Phylobayes MPI 1.8c (Lartillot et al. 2013) and a maximum-likelihood (ML) framework with IQ-Tree 2.1.2 (Nguyen et al. 2015). Phylobayes nucleotide analyses were run for 10000 cycles using the GTR exchange rates and the CAT profile mixture. Convergence, mixing and effective samples size were checked by examining the relative differences amongst chains (< 0.2), as well as using Tracer 1.7 (Rambaut et al. 2018). IQ-Tree nucleotide analyses were run under an edge-linked model with each partition having an independent evolutionary model selected with PartitionFinder 2. Node support was assessed using 1000 ultrafast bootstrap replicates (Hoang et al. 2018). The protein phylogenetic analyses were run with the general metazoan mitochondrial amino acid substitution model (Mt-ZOA) (Rota-Stabelli et al. 2009) for both Phylobayes and IQ-tree with the same settings as for nucleotides. All phylogenetic analyses were carried out using the computational infrastructure available at the CIPRES Science Gateway (Miller et al. 2010).

Data accessibility

All mitochondrial genomes are available in NCBI GenBank, accession numbers OR233270–OR233282, as well as on Figshare (DOI: 10.6084/m9.figshare.22753487).

Results

Mitochondrial genomic structure

All samples yielded high-quality reads that could be assembled into complete mitochondrial genomes containing the expected number of 13 protein-coding genes, large and small-subunit rRNA and 22 transfer RNAs. Mitogenome length varied between 14,694 bp (C. ischnus) and 18,195 bp (G lacustris); see Table 1 for length of all mitogenomes. In most cases, the inferred gene order is similar between the native and invasive Ponto-Caspian species. The most observed difference is a translocation of the tRNA-E and tRNA-R, which aligns with previous observations, based on less extensive taxonomic datasets (Bojko 2020; Mamos et al. 2021). However, there are also a few rather contrasting patterns of variation between the native and invasive groups (Fig. 1). The native species exhibit three general patterns: (1) minor translocations (swaps) between tRNAs (tRNA-N, tRNA-E and tRNA-R) as observed in G. roeselii and G. varsoviensis; (2) significant translocations of multiple tRNAs and the NADH dehydrogenase 1 gene (nad1) in P. quadrispinosa and S. ambulans; (3) a polarity switch of the tRNA-F in S. ambulans. The gene arrangement in the Ponto-Caspian gammarids is identical in all studied species, except C. warpachowskyi, which shows a significant departure with the translocation of six tRNAs. The gene order in the Ponto-Caspian alien corophild C. curvispinum is identical to that of most native species (Fig. 1). In general, the gene arrangements appear to follow phylogenetic relationships.

Nucleotide composition

Multivariate analyses indicate a significant differentiation with respect to nucleotide composition between the native and invasive species. The PCA scatterplot indicates a modest overlap between native and invasive groups in multivariate space, with the first two axes explaining 98% of the observed variance (Fig. 2A). The separation is further confirmed by PERMANOVA testing which indicates significant differences between the two groups (F = 6.257, p = 0.01). The invasive species are generally associated with a higher AT content, while native species with a higher GC content, although with a large variation in GC content of native species, is observed. Further univariate comparisons using Mann-Whitney tests reveal that invasive species have a significantly higher AT and significantly lower GC content than the native species (mean AT% native = 68.86, mean AT% native = 60.99, z = 3.04, p = 0.001; mean GC% invasive = 38.97, z = 3.48, p = 0.001) (Fig. 2B).

Phylogenetic analyses

Phylogenetic analyses revealed congruent relationships between methods (BI and ML) and datasets (nucleotides and amino acids) (Fig. 3). Disagreements were observed only at unsupported nodes. The native European inland water species are phylogenetically diverse, interspersed between two main superfamilies, the Gammaroidea and Crangonyctoidea. Although the alien Ponto-Caspian species also belong to two main superfamilies, Gammaroidea and Corophioidea, the gammarids form a strongly-supported monophylum. Our analyses reveal for the first time the phylogenetic position of *P. quadrispinosa*, confirming it as a sister species to the Baikal endemic *P. kessleri* and ultimately part of the Baikal Lake acanthogammarid radiation.

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Gammarus fossarum	Gammarus jazdzewskii	Gammarus wautieri	Gammarus pulex	Gammarus roeselii	Gammarus lacustris	Gammarus varsoviensis	Gammarus duebeni	Pallaseopsis quadrispinosa	Echinogammarus berilloni	Dikerogammarus haemobaphes	Dikerogammarus villosus	Dikerogammarus bispinosus	Obesogammarus crassus	Pontogammarus robustoides	Chaetogammarus ischnus	Chaetogammarus warpachowskyi	Pectenogammarus veneris	Synurella ambulans	Chelicorophium curvispinum	Putative pancrustacean ground pati	areviscale translocated genes
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native	Ponto-Caspian alien	J						Pallaseidae				Ponto-Caspian	radiation		7			Crangonyctidae	Corophildae		Figure 1 Comparison of mitochot





Figure 2. Differentiation of native European and invasive Ponto-Caspian amphipod species with respect to nucleotide composition across the entire mitochondrial genome. A) PCA scatterplot depicting multivariate differentiation across all four nucleotides; B) boxplots comparing AT and GC content between native and invasive species.

Discussion

The patterns of mitogenomic rearrangements observed in this study are consistent with the diversity that has been observed in other amphipod clades, ranging from major differentiation at generic levels to highly conserved between divergent clades (Bauzà-Ribot et al. 2009; Stokkan et al. 2016; Li et al. 2019; Zapelloni et al. 2021). The mitogenomic phylogenetic relationships obtained herein are also in agreement with other phylogenetic studies, based on nuclear and mitochondrial markers (Copilaş-Ciocianu et al. 2020).

Our study reveals that the native inland European amphipods can exhibit substantial differences with respect to mitogenomic organisation, while the alien Ponto-Caspian species are more conservative. This is not unexpected given the greater phylogenetic disparity amongst the native species. However, the organisation patterns seem not always to be phylogeny driven. For example, C. curvispinum, which is distantly related to the other focal species in this study, exhibits a conserved gene arrangement, identical to that of most native species. On the other hand, P. quadrispinosa is more closely related to other native gammarids, yet it diverges significantly with respect to gene order. In fact, the gene order of *P. quadrispinosa* is identical to that of its congener from Lake Baikal, P. kessleri (Romanova et al. 2016). Our study confirms for the first time with molecular data the phylogenetic position of this species, which is a glacial relict that has almost become extinct in Central Europe due to climate warming and eutrophication (Meßner and Zettler 2021; Arbačiauskas et al. 2022). The peculiar mitogenomic structure of Pallaseopsis is outstanding even amongst other Baikalian amphipods (Rivarola-Duarte et al. 2014; Romanova et al. 2014, 2016, 2021), possibly reflecting intense periods of selection (Naumenko et al. 2017; Romanova and Sherbakov 2019).



Figure 3. Amino acid Bayesian phylogeny, based on 13 mitochondrial protein-coding genes depicting the evolutionary relationships amongst the focal taxa (highlighted with colour). Native European surface-dwelling species are shown with green shading, while invasive Ponto-Caspian species are in purple. Stars indicate taxa sequenced in this study. Green circles indicate nodes that received strong support in all analyses. Nodes with numbers received moderate to strong support. Numbers above nodes indicate statistical support (posterior probabilities—PP; ultrafast bootstrap—UFBS) for amino acid-based trees; below nodes for nucleotide-based trees. Nodes that are not annotated received weak/no support (PP < 0.5, UFBS < 50%). Inset photographs from top to bottom: *G. fossarum, P. quadrispinosa, C. warpachowskyi* and *S. ambulans* (D. Copilaș-Ciocianu).

The native crangonyctid *S. ambulans* is phylogenetically very distant from the native gammarids and its mitogenomic structure is highly distinct as well. Several tRNAs and the nad1 gene in *S. ambulans* have undergone translocations. Moreover, we detected a switch to a positive polarity of the tRNA-F gene, which normally is found on the minusstrand in amphipods. This pattern is partially phylogeny-driven, because the available mitogenomes of other crangonyctids seem to be generally conserved, but in some cases can show significant transpositions (Benito et al. 2021). The remaining native gammarids
(*Echinogammarus*, *Gammarus* and *Pectenogammarus*) possess a conserved mitogenomic structure, with the main differences involving minor translocations of tRNAs, particularly between tRNA-E and tRNA-R. The mitogenome of *G. varsoviensis* exhibits a previously-unknown translocation of the tRNA-N, situated between tRNA-R and tRNA-E.

The alien Ponto-Caspian gammarids exhibit a more conserved gene order than their native counterparts. Apart from the phylogenetically distant *C. warpachowskyi*, all species have identical mitogenomic structures. They differ from native species due to a swap between tRNA-E and tRNA-R, a pattern observed in previous studies with less taxonomically comprehensive datasets (Bojko 2020; Mamos et al. 2021). However, we demonstrate that this does not apply to all Ponto-Caspian gammarids, as *C. warpachowskyi* exhibits significant differences from this pattern with translocations of six tRNAs. This deviation may be attributable to its phylogenetic position, since this species is more distantly related to other Ponto-Caspian gammarids and should be assigned to a new genus (Copilaş-Ciocianu et al. 2022; Copilaş-Ciocianu et al. 2023b). Sequencing additional mitogenomes from Ponto-Caspian gammaroidean species will likely uncover further gene rearrangement patterns, as only seven of 82 species have been sequenced so far (Copilaş-Ciocianu and Sidorov 2022).

Aside from gene order, we discovered substantial differentiation in nucleotide composition between native and invasive Ponto-Caspian species. Invasive species possess significantly more AT-rich mitogenomes than natives, while natives exhibit higher GC content. This finding suggests that invasive species may have longer non-coding regions or that native species have protein-coding genes with higher GC content, which overall indicates more compact mitogenomes in the latter (Romanova et al. 2020; Sun et al. 2022). The relationship between this differentiation in GC content and invasion ability remains unclear, but it could potentially open new avenues for research.

With respect to phylogenetic relationships, our study is in broad agreement with previous molecular work. We further confirm the phylogenetic disparity of the native species pool, mirroring previous multilocus phylogenies (Copilaş-Ciocianu et al. 2020). Specifically, we corroborate the polyphyly of the genus *Gammarus* by recovering the two main Baikal gammarid radiations (Acanthogammaridae and Micruropodidae+Macrohectopidae) as nested within it (Hou and Sket 2016; Romanova et al. 2016; Naumenko et al. 2017; Mamos et al. 2021) and, for the first time, confirm with molecular data that *P. quadrispinosa* is indeed of Baikalian origin. Our trees also show that crangonyctids are more distantly related to gammarids than the current morphology-based classification suggests (Lowry and Myers 2017). Regarding the invasive Ponto-Caspian species, we confirm the monophyly of the gammaroids (Hou et al. 2014) and reveal for the first time the position of *C. curvispinum*.

Conclusion

Our comparative analyses highlight substantial differentiation between the mitogenomes of native European and invasive Ponto-Caspian amphipod crustacean species. Native species, being more phylogenetically diverse, display varied mitogenomic configurations and higher GC content compared to the less phylogenetically dispersed invasive species, which exhibit highly conserved gene order and increased AT content. We propose that these differences are not solely determined by phylogeny, as gene order conservation can vary across phylogenetic depths, but may also be shaped by other evolutionary factors including selective pressure. Exploring the biological implications of these mitogenomic distinctions between native and invasive amphipods may provide insight into the adaptive mechanisms that contribute to invasion success.

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Supplementary material I

Origin of samples and mitochondrial genomes used in this study

Authors: Jan-Niklas Macher, Eglė Šidagytė-Copilas, Denis Copilaș-Ciocianu Data type: table (excel file)

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RESEARCH ARTICLE



Weed wide web: characterising illegal online trade of invasive plants in Australia

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Abstract

Invasive plants seriously impact our environmental, agricultural and forestry assets, and the ornamental plant trade is a major introduction pathway. The variety and extent of the ornamental plant trade is growing in reach and is increasingly facilitated by the internet (i.e., through e-commerce). A lack of surveillance and regulation of e-commerce has resulted in invasive species being widely traded on these platforms. Here, we investigated the extent of illegal trade in invasive plant species in Australia by collecting advertisements found on a popular public e-commerce website. Across a 12-month period we collected a total of 235,162 plant advertisements. From 10,000 of these advertisements (4.25% of total advertisements) we found 155 plant taxa advertised online that were prohibited to trade in at least one Australian State or Territory (12.5% of Australia's total prohibited plant taxa). We detected 1,415 instances of invasive plants advertised, of which 411 breached local jurisdictional (i.e., State or Territory) laws. Opuntia cacti and invasive aquatic plants were traded in the greatest quantities. A variety of uses for plants prohibited to trade were reported by the sellers, with aquatic uses being the most popular (i.e., water filtering and habitat for aquatic animals). We used generalised linear mixed-effects models to test the effect of prohibiting the sale of invasive plants on the quantity and price of online advertisements. Despite Australia's strict internal biosecurity regulations, we found that trade prohibitions had no influence on the quantity and price of trade in illegal invasive plants. Given this, and the extent of illegal invasive plants traded, we believe increased monitoring and regulation of online plant trade is warranted. We demonstrate that targeted searches using string matching is an effective tool for detecting e-commerce trade of invasive species. However, to obtain the most optimal outcomes, regulations should be coupled with increased cooperation from e-commerce

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platforms and public awareness campaigns. Future weed risk assessments should consider online trade as a key factor in the long-distance dispersal and propagule pressure of a plant. Jurisdictions would also benefit from greater alignment on plant trade prohibitions and revision of associated compliance policies.

Keywords

Aquatic weeds, biosecurity, e-commerce, Opuntia, ornamental plants, prevention, surveillance, web scraping

Introduction

Invasive plants can cause serious negative impacts to biodiversity, human health, and primary resource industries (Pyšek et al. 2020; Ward et al. 2021). The largest vector of new plant introductions and invasions is the global trade of ornamental plants, which is continually growing in both reach and quantity (Weber et al. 2008; Dodd et al. 2015; Faulkner et al. 2016; van Kleunen et al. 2018; Arianoutsou et al. 2021; Beaury et al. 2021; Rojas-Sandoval et al. 2022). Within this global trade, a pathway of serious concern is trade facilitated by the internet, hereafter termed e-commerce (Derraik and Phillips 2010; Lenda et al. 2014; Humair et al. 2015). E-commerce platforms facilitate long distance dispersal of invasive species and can often circumvent regulations (Giltrap et al. 2009; Derraik and Phillips 2010; Magalhães and Avelar 2012; Lenda et al. 2014; Humair et al. 2015; Beaury et al. 2021). As a result, e-commerce has proven challenging to monitor and enforce for biosecurity agencies (Derraik and Phillips 2010; Lavorgna and Sajeva 2021). Many invasive plant species are being traded online despite legislative regulations (Humair et al. 2015; Munakamwe and Constantine 2017; Beaury et al. 2021). Without intervention, it is predicted that online trade will lead to further invasive plant incursions (Humair et al. 2015; Peres et al. 2018; Beaury et al. 2021).

Australia has a highly endemic floral community that has been severely impacted by plant invasions (Broadhurst and Coates 2017; Bradshaw et al. 2021). Strict importation measures and risk assessment processes have been implemented by the Australian government to prevent the arrival of new alien-invasive plants (Pheloung et al. 1999; Walton 2001; Keller et al. 2007; Simberloff et al. 2013). Even so, Australia already has more than 29,000 introduced alien-plant species (Gallagher and Leishman 2014). There are also native Australian plants which have become invasive outside their indigenous range (Rose and Fairweather 1997; Morgan et al. 2002; O'Loughlin et al. 2015). Where plant species become invasive, or there is potential to be invasive, state and territory governments ('jurisdictions' hereafter) have the main responsibility for their management and control. A common control measure used by jurisdictions is to 'declare' invasive plant taxa in legislation as prohibited to trade within jurisdictional borders (simply 'declared plant' hereafter); with 1,236 taxa declared in one or more jurisdictions across Australia. These taxa are declared because they pose significant risks of environmental, economic and/or social impacts to natural ecosystems, agricultural and forestry production, and human communities. While legislation differs slightly between jurisdictions, generally it is prohibited to supply, sell, or transport declared plants, with fines issued for offences. However, e-commerce websites could circumvent traditional

enforcement measures by trading without physical stores, sending plants by mail, or having buyers collect plants from private residences, resulting in a poorly regulated sector of the horticultural market (Munakamwe and Constantine 2017). Screening for invasive plants entering the country is also challenging due to the high volume of incoming international mail (Australian National Audit Office 2014). Therefore, surveillance of e-commerce is an essential tool for detecting and preventing plant invasions (Humair et al. 2015; Lavorgna et al. 2020; Duncan 2021; Stoett and Omrow 2021; Whitehead et al. 2021). E-commerce websites where members of the public post plant advertisements are particularly difficult to monitor. Some efforts have been made to monitor this trade within Australia, however the focus has been limited by time and resources to a handful of problematic species (Munakamwe and Constantine 2017).

To investigate the current invasion risk of e-commerce plant trade within Australia (i.e., internal trade, not international shipments into Australia), we applied web-scraping technology to monitor and record plant trade advertisements on a popular Australian e-commerce website over the course of one year. We investigated five research aims: (i) determine what proportion of plants advertised are prohibited to trade; (ii) determine the quantity and taxonomic composition of declared plants traded; (iii) determine whether current regulations reduce trade quantity or influence the price of declared plants in jurisdictions which prohibit trade versus those that permit trade; (iv) characterise the most frequently traded declared plants; and (v) document advertised plant uses to inform our understanding of the desire for declared plants. We seek to provide a clearer picture of the present risk of e-commerce trade and whether prescriptive laws reduce invasive plant trade. These results will help inform future policy decisions regarding the monitoring and prevention of invasive species occurring in the Australian plant trade.

Methods

Compiling Australia's declared plants

In order to investigate the trade of invasive plants online, we compiled a list of declared plants in Australia. These declared plants are prohibited from trade under jurisdictional biosecurity legislation because of their current or potential impact as invasive species (Parsons and Cuthbertson 2001). Declaration is usually based on an analysis of weed risk using various post-border weed risk management systems (Virtue et al. 2006). Jurisdictional declarations can include Australian native plant species that have invaded beyond their indigenous range, for example a Western Australian species that is invasive in eastern Australia. Hence declared native plant species are included in this study. To assemble a comprehensive list of declared plants, we used sources relevant to Australia's eight main jurisdictions (i.e., six states plus Northern Territory and Australian Capital Territory), including government websites, online databases, legislative acts, and gazettes (see Suppl. material 1 for complete list of sources). Our compiled list of declared plants and relevant legislation was reviewed and endorsed by appropriate jurisdiction-al officials through the Weeds Working Group of the Australian intergovernmental

Environment and Invasives Committee. We standardised the taxonomy of the declared plants using the Global Biodiversity Information Facility taxonomic database (GBIF 2021). Our finalised list of declared plant taxa contained 1,236 defined taxa comprising 1,178 species, 6 subspecies, and 5 varieties, as well as 47 declared genera. Twenty-two of the declared plant species are recognised as native by the Australian Plant Census and 2 species have uncertain native status (Australian National Herbarium 2023).

E-commerce platform selection and building web scrapers

We followed established protocols to select e-commerce websites to monitor for sales of plants (Stringham et al. 2020). Specifically, we conducted a systematic web search of invasive plant species names (common and scientific) with an appropriate phrase e.g., "Vinca major for sale Australia" and "Periwinkle for sale Australia". To optimise the search effort in selecting e-commerce websites for further investigation, we created a short-list of declared species known to be popular in horticulture (Suppl. material 2) (Nursery & Garden Industry Australia 2009). A total of 38 nursery websites and 4 public e-commerce websites were reviewed. We defined nursery websites as private online businesses. Public e-commerce websites host online classifieds where members of the public can post personal advertisements. We found plants considered to be invasive on nursery websites, but we did not find any that were declared in the jurisdiction the nursery was located in (i.e., no prohibited advertisements). In contrast, our initial investigations of public e-commerce yielded many prohibited advertisements for declared plants. Alongside our internet search, we consulted with biosecurity officers from each jurisdiction who had experience monitoring the online plant trade. They identified public e-commerce websites over private nursery websites as their primary concern, citing regular detections of declared plants on the former in their own investigations. The risk of public e-commerce is an under-assessed aspect of the ornamental plant trade as it is difficult to monitor and regulate. Based on this recommendation and the findings of our web search, we concentrated our study on one highly popular public e-commerce website. This allowed us to construct a reliable and consistent web scraper for a popular e-commerce website that included seller location data and which frequently traded declared plants, based on expert opinion and our preliminary search. This website hosts trade within Australia and is not specific to ornamental plants. However, the website has a 'plant' category from which we collected advertisements. Sellers advertise plants, and sales are conducted through private exchanges between traders either online, over the phone, or in person. Therefore, it is important to note that we could not determine how many plants were actually sold from the data we collected. Similarly, we could not determine how many advertisements were relisted plants that had previously failed to sell. We have kept the identity of this website anonymous in accordance with our ethics approval (Ethics approval H-2020-184). Personal and identifiable information of traders is available on this website and while publicly available our ethics involve taking a cautious approach to avoid revealing behaviour which may have legal ramifications. Additionally, identifying the website could alter the behaviour of traders which would reduce the value of ongoing surveillance research (Stringham et al. 2020).

To collect online advertisement data, we constructed a custom web scraper in Python Programming Language (version 3.8.1; Python Software Foundation 2020) using the libraries bs4 (Richardson 2020), requests (Reitz 2020), and selenium (Selenium Main Repository 2020). The web scraper ran daily and collected all advertisements from the designated plant category of the website. Plant advertisement data was stored on a local SQL database. For this study, we explored 12 months of plant advertisements between 1 February 2020 and 31 January 2021. Duplicate collections of advertisements were common because the web scraper ran on a daily basis. We removed these duplicate advertisements based on a unique listing identifier generated by the website. This resulted in 235,162 unique advertisements that did not provide a seller location, leaving us a dataset of 233,694 advertisements.

Sampling and detecting declared plant trade

The data we collected were not immediately ready for analysis because the advertisements from the website were composed of free-form text boxes completed by the users, and thus the taxonomic names could not be automatically retrieved (i.e., no standardization in names). Identification of plants was conducted manually using text and pictures, provided by the seller, which was a time-consuming process. Subsequently, we explored a subset of the advertisements. For our study, we extracted two samples of 5,000 advertisements each. The first sample was a random sample of all plants traded stratified by jurisdiction. For the second sample we utilised natural language processing to focus specifically on detecting declared plants.

The first sample was untargeted; it sampled from all the advertisements we collected and did not intentionally target declared plants. This sample was stratified by jurisdiction with 625 unique advertisements randomly sampled from each jurisdiction, providing 5,000 advertisements in total. We used this dataset to estimate the underlying proportion of declared plant trade in each jurisdiction and to compare the effectiveness of our targeted sampling method.

For the second sample we targeted declared plant advertisements. Our objective was to identify frequently traded declared plants, and capture the composition of declared plants traded. We aimed to capture declared plants traded anywhere in Australia regardless of whether they were advertised in a prohibited jurisdiction. This was to capture the full extent of declared plant trade in Australia. To do this we used string matching to generate a targeted sample aimed at detecting declared plant advertisements (Stringham et al. 2021). String matching is a natural language processing method of finding a sequence of characters, called a string, that match a given character pattern. In our case the character patterns were the scientific and common names of declared plants. In total, we used 10,573 names to search for the 1,236 declared taxa within the text of collected advertisements. We initially sourced common names from jurisdiction legislation, followed by broader internet searches if necessary (Suppl. material 1) (Shepherd et al. 2001). We cleaned names by removing parentheses and punctuation, converted to lower case, and also pluralised and singularised the names. Based on findings by Munakamwe and Constantine (2017), we included common terms for some aquatic species. String matching helped reduce the number of advertisements down to a more manageable data set with a higher probability of detecting declared plants. However, common and generic plant names are non-specific and can be shared by many species. This resulted in false positives in the targeted sample. Our pilot investigation revealed some frequent false positives due to the inclusion of certain broad search terms (e.g., 'lily' returned many non-target species). We created a list of match exceptions to remove the bulk of the false positives (Suppl. material 3). Therefore, if an advertisement contained the word 'lily' and contained a match exception such as 'peace lily' (a non-target species) it would be removed, but an advertisement for 'arum lily' would remain. This approach helped us to reduce the number of false positives while retaining the use of certain generic search terms. Out of 233,694 total advertisements, text in the title or description matched to 12,751 advertisements for declared plants. From this, we took a sample of 5,000 unique advertisements. Given our interest in characterising the legality of online trade across Australian jurisdictions, we stratified the sample by jurisdiction. Three jurisdictions had substantially fewer advertisements: Australian Capital Territory, Northern Territory, and Tasmania (Table 1). To help capture trade from these three smaller jurisdictions all advertisements that matched declared plant search terms were analysed. The remaining jurisdictions were randomly sampled until 5,000 unique advertisements was reached (Table 1).

We cleaned the sampled datasets by identifying the plants in each advertisement using photos and text provided by the seller. Advertisements would often contain multiple species for sale so we recorded each plant species (or lowest taxonomic rank possible) as a separate identification within an advertisement. We recorded the price and quantity for each plant identified, and the location of the advertisement. It is important to note that recorded locations were seller locations and not where a plant may have been transported to after it had been purchased. Predominately, advertisements were for live plants, however we also captured trade of seeds and other propagules. We documented and categorised advertisements that stated uses for plants when specified by sellers (i.e., used for purposes other than as a live ornamental plant, including propagules).

Once we identified the plant taxa in the advertisements, we cross referenced them with our dataset of 1,236 declared plants. We recorded the number of plant taxa identified and how many were declared plants. We used species accumulation curves to assess how well our samples captured the diversity of plant taxa and declared plant taxa traded online. We measured the number of advertisements containing declared plants and identified advertisements that were prohibited (i.e., the advertisement contained a plant that was declared in the jurisdiction where it was advertised). However, multiple declared plant taxa could appear in a single advertisement. To account for this, we also recorded each detection of a declared plant taxon in any single advertisement. To help explain these different types of trade observations an example with term definitions is provided in Fig. 1. By using these observation metrics, we were able to capture prohibited trade of a declared plant and the broader extent of its trade within Australia.

Table 1. The number of advertisements collected and sampled from an e-commerce website stratified by jurisdiction. The table provides the number of advertisements from: (i) 12 months of web scraping (Total dataset); (ii) the untargeted sample (Untargeted); (iii) the string-matching for declared plant taxa (Matched); and (iv) the targeted sample (Targeted). The targeted sample was weighted to better capture trade in three jurisdictions with comparatively lower quantities of matched advertisements: Australian Capital Territory, Northern Territory, and Tasmania (* indicates weighted samples). All advertisements that matched search terms for declared plants in these jurisdictions were cleaned. The remaining advertisements were sampled randomly across the remaining jurisdictions to total 5,000 advertisements.

Jurisdiction	Total dataset	Untargeted		Matched	Targeted
Australian Capital Territory (ACT)	7,362	625	String matching using	420	*420
New South Wales (NSW)	64,641	625	declared plant search	3,351	1,031
Northern Territory (NT)	859	625	$terms \rightarrow$	66	*66
Queensland (Qld)	48,909	625		2,893	948
South Australia (SA)	21,121	625		1,073	539
Tasmania (Tas.)	5,991	625		308	*308
Victoria (Vic.)	41,186	625		2,567	921
Western Australia (WA)	43,625	625		2,073	767
Total	233,694	5,000		12,751	5,000



Figure 1. A diagram explaining the terms we used to define the different types of plant trade observations. This example shows two advertisements and two species of declared plant (plants prohibited to trade in a given jurisdiction). The number of observations for each term in this scenario are provided in parentheses. In the 'prohibited jurisdiction' there is one advertisement with two plant species, both species are prohibited to trade in this jurisdiction. One of these plant species is sold by itself in the 'permitted jurisdiction'. In this case we refer to it as a declared plant, but it is permitted to trade in that jurisdiction.

Analysis of prohibited trade on quantity and price

We used generalised linear mixed-effects models to test whether prohibited trade had an effect on the trade quantity and price of declared plants. These models considered declared status as the binary explanatory variable and taxa identity as a random effect

(i.e., random intercept). For quantity, we hypothesised fewer declared plants are advertised in jurisdictions that prohibit their trade compared to jurisdictions that permit their trade. We based our rationale on the notion that laws prohibiting trade would reduce the number of advertisements online. For price, we hypothesised that in jurisdictions that prohibit trade, prices for declared plants would be higher compared to jurisdictions that permit trade. Our rationale was that laws prohibiting trade would result in an increased price to offset their risk; i.e., buyers paying a premium for prohibited plants. We measured the performance of the models using Nakagawa and Schielzeth's conditional R-squared (Rc2) (Nakagawa and Schielzeth 2013). For all models, we used the targeted dataset, which had the greater number of total declared advertisements compared to the untargeted. For these models, we removed nationally declared taxa, i.e., taxa declared in all jurisdictions (n = 130 taxa remaining for quantity comparison). In the quantity models, we defined quantity as the proportion of advertisements within each jurisdiction's sample. This approach was to account for differing sample sizes among jurisdictions in the target dataset (see Table 1 for sample sizes). For analysing price differences, we used unit prices (price per plant) gathered from the targeted and untargeted datasets. Further, for these price models, we excluded taxa with fewer than two advertisements in each legality category (i.e., prohibited or permitted); this limited the model to 20 taxa. There were two factors that contributed to this reduction. Firstly, a price per plant could not be determined for many advertisements. Either no clear price was provided or plants (particularly aquatic species) were priced by inconsistent container volumes (i.e., \$5 for a full take-away container). Secondly, for some plants price data was absent from a legal category (i.e., no prices recorded in either a prohibited or permitted jurisdiction).

We took an additional approach to assess and visualise the difference in quantity and price by exploring the distribution of differences in quantity and price. We calculated the difference of mean quantity and price of each declared plant taxon traded in prohibited jurisdictions compared to permitted jurisdictions (i.e., the mean quantity of taxon A pooled across all prohibited jurisdictions minus the mean quantity of taxon A pooled across all permitted jurisdictions). We used this distribution to determine the degree that prohibited trade affected trade quantity and price, where a distribution centred around zero with low variation suggests little to no influence.

Data and software resources

We conducted data analysis and visualisation using the R software environment for statistical and graphical computing (version 4.1.1; R Core Team 2022) and used the following packages for our analyses. We verified taxonomy by using the 'taxize' package (Scott Chamberlain 2013) and to acquire information from the Global Biodiversity Information Facility taxonomic database. Plant search terms were pluralised using the 'pluralize' package (Rudis and Embrey 2020) and string matching was performed using the 'stringr' package (Wickham 2019). Collected data was accessed from MySQL database using the 'DBI' package (Wickham and Müller 2022). Regression model

coefficients were summarised and extracted using the 'broom' package (Robinson et al. 2021). Shapefiles were obtained from the Australian Bureau of Statistics (2021) and visualised using the 'sf' package (Pebesma 2018). Species accumulation curves were calculated using the 'vegan' package (Oksanen et al. 2020). The following packages were used for handling and manipulating data: 'tidyverse' (Wickham et al. 2019), 'dbplyr' (Wickham et al. 2021), 'lubridate' (Grolemund and Wickham 2011), and 'sampler' (Baldassaro 2019). To create and assess models we used: 'lme4' (Barton 2020), 'lmerT-est' (Kuznetsova et al. 2017), and 'MuMIn' (Bates et al. 2015) packages. The following packages were used for data visualisation: 'tidyverse' (Wickham et al. 2019), 'cowplot' (Wilke 2020), 'ggalluvial' (Brunson and Read 2020), 'ggrepel' (Slowikowski 2021), 'ggpubr' (Kassambara 2020), and 'scales' (Wickham and Seidel 2022). The data underpinning the methods and analysis of this study have been deposited on the Figshare Repository at https://doi.org/10.6084/m9.figshare.22493944 (Maher et al. 2023).

Results

Overall richness, trade proportion, and detection rate

From the 10,000 advertisements we examined (i.e., 5,000 each for the untargeted and targeted samples), we made 13,619 plant identifications (average c. 1.4 identifications per advertisement). We identified 1,777 unique plant taxa (Fig. 2a) of which 78 were declared plants prohibited to trade in the jurisdictions where they were advertised (c. 6% of declared plants). A further 77 declared plants were advertised legally in juris-



Figure 2. Accumulation curves of plant taxa identified from sampling 10,000 online advertisements **A** accumulation curve of all plant taxa identified. There were 1,777 taxa observed from 10,000 advertisements **B** accumulation curves of declared plant taxa identified. The red line represents a targeted sample that utilised search terms to locate declared plant advertisements and the blue line represents an untargeted sample that did not use search terms (i.e., random sampling). There were 155 declared taxa identified in 1,415 detections of declared plants.

dictions that do not prohibit their trade. This brought the overall number of declared plants traded to 155 taxa (c. 12.5% of all declared plants in Australia) (Fig. 2b). We did not observe any of the species accumulation curves approaching a clear limit (Fig. 2).

From the 10,000 advertisements examined, we made 411 prohibited detections (from 374 advertisements) within 1,415 total declared detections (from 1,296 advertisements). From our untargeted sample, we found 59 prohibited advertisements (c. 1%) and 150 total declared advertisements (detection rate of 3%). In comparison, our targeted sample contained 328 prohibited advertisements (c. 7%) and 1,183 total declared advertisements (detection rate of c. 24%) (Fig. 3). New South Wales (NSW) and Victoria (Vic.) are the most populous jurisdictions in Australia (Australian Bureau of Statistics 2020) and had the greatest number of total declared advertisements. Western Australia (WA) declares the greatest number of plant taxa of any Australia jurisdiction (877 plant taxa) and had the greatest number of prohibited advertisements (Fig. 3).



Figure 3. The number of advertisements for declared plants detected on an e-commerce platform over a 12-month period. These detections were made from a sample of 5,000 advertisements that had been matched to search terms for declared plants (i.e., targeted sample) **A** the number of prohibited declared plant advertisements detected within the jurisdiction (i.e., prohibited in that jurisdiction, refer Fig. 2). The colour refers to the percentage of advertisements that were prohibited **B** the total number of declared plant advertisements detected in that jurisdiction that are declared anywhere in Australia. The colour refers to the percentage of advertisements take advertisements that contained declared plants **C** the 2020 resident population (Population) and number of plant taxa declared in each jurisdiction (Plants declared). Population data was sourced from Australian Bureau of Statistics (2020).

Influence of trade prohibition on quantity and price

The generalised linear mixed-effects models revealed no statistically significant effect on the quantity and price of declared plants between jurisdictions that prohibited trade and those that did not. The model for quantity had a *p*-value of 0.58 for the quantity coefficient, with a sample size of 1040, which covered 130 declared taxa (quantity coefficient estimate = $-0.000266 \pm SE = 0.000479$; *t* = -0.56; Rc2 = 0.32). The model for price had a *p*-value of 0.13 for the price coefficient, with a sample size of 652, covering 20 declared taxa (price coefficient estimate = $-6.25 \pm SE = 4.11$; *t* = -1.52; Rc2 = 0.24).

For over 80% (104/130 taxa) of declared taxa analysed, the mean difference in the number of advertisements between prohibited and permitted jurisdictions was less than one advertisement (Fig. 4). The declared plants with the greatest mean differences were *Drimia maitima* (mean absolute difference c. 5 plants) which had higher quantities in



Figure 4. Distribution of the mean difference in the number of advertisements for declared plant taxa between prohibited and permitted jurisdictions. The black curve overlaying the histogram represents the cumulative distribution of mean differences in advertisement quantities. A positive mean difference translates to comparatively more advertisements in prohibited jurisdictions and fewer in permitted jurisdictions. A negative mean difference translates to comparatively more advertisements in prohibited jurisdictions and fewer in permitted jurisdictions. The distribution represents 130 plant taxa and each bar represents one advertisement. We removed taxa that are declared in all jurisdictions and those with fewer than two advertisements in each legality category (i.e., prohibited or permitted) as there was nothing to compare against.

prohibited jurisdictions, and *Opuntia ficus-indica* (mean absolute difference c. 11 plants) with higher quantities in permitted jurisdictions. We found far fewer advertisements for declared plants in the untargeted sample compared to the targeted sample (Table 2). Across jurisdictions the proportion of prohibited advertisements was c. 0.2–2% and total declared advertisements was c. 1–5% in the untargeted sample (Table 2). The highest proportion of prohibited advertisements was observed in South Australia (SA) and NSW for the untargeted sample. In comparison, the detection rate in the targeted sample rose to c. 3–15% for prohibited and c. 16–28% for total declared advertisements was observed in Northern Territory (NT), SA, and WA for the targeted sample (Table 2 and Fig. 3).

The distribution of plant prices was similar across jurisdictions, typically ranging from \$5 to \$40 for a potted plant (Australian dollars; AUD) (Suppl. material 4). On average, prices were only \$1.25 more in prohibited jurisdictions with 60% (12/20 taxa) of observed taxa having a mean price difference within \$5 (Suppl. material 5). However, the sample size for the price model was greatly reduced compared to the quantity model, with only 20 declared plant taxa included.

Most frequently traded declared plants and advertised uses

The most frequently advertised declared plants were *Opuntia* cacti and aquatic weeds (Fig. 5). The declared plant with the greatest number of prohibited advertisements was *Opuntia microdasys* (bunny ears cactus) (Fig. 5b). Other *Opuntia* species were frequently traded, including *Opuntia monacantha* (drooping prickly pear) and *Opuntia*

Table 2. Summary of advertisements for declared plants in Australia's eight jurisdictions. Results are presented from two samples collected across 12 months of e-commerce activity. The untargeted sample represents a consistent number of plant advertisements sampled for each jurisdiction, based on the location of the seller. The targeted sample is a focused search for advertisements matching declared plant search terms, resulting in a variable number of advertisements sampled for each jurisdiction. The 'Prohibited' column indicates the count of advertisements (Ads) containing plants declared within the respective jurisdiction where the advertisement is located. The 'Total Declared' presents the number of advertisements (Ads) containing plants declared anywhere in Australia. The percentages (%) are calculated based on these observations and the respective sample sizes, with darker colours for higher relative percentages. The sample sizes represent the total number of advertisements considered in each jurisdiction.

Jurisdiction	Untargeted Sample					Targeted Sample				
-	Prohibited		Total declared		Sample	Prohibited		Total declared		Sample
	Ads %		Ads	%	size	Ads	%	Ads	%	size
Australian Capital Territory (ACT)	7	1.12	19	3.04	625	13	3.10	89	21.19	420
New South Wales (NSW)	11	1.76	19	3.04	625	77	7.47	297	28.81	1031
Northern Territory (NT)	9	1.44	32	5.12	625	10	15.15	17	25.76	66
Queensland (Qld)	7	1.12	13	2.08	625	27	2.85	155	16.35	948
South Australia (SA)	13	2.08	21	3.36	625	62	11.50	139	25.79	539
Tasmania (Tas)	1	0.16	10	1.60	625	20	6.49	78	25.32	308
Victoria (Vic)	9	1.44	28	4.48	625	36	3.91	249	27.04	921
Western Australia (WA)	2	0.32	8	1.28	625	83	10.82	159	20.73	767
Total	59	1.18	150	3.00	5000	328	6.56	1183	23.66	5000



Figure 5. Invasive plants most frequently advertised on an e-commerce platform during a 12-month period. These plants are prohibited to trade in one or more Australian jurisdictions (i.e., declared plants) **A** the size of the declared plant photos is approximately scaled by their relative frequency in trade **B** lists the 10 declared plants that were most frequently advertised in jurisdictions where they are prohibited to trade (i.e., advertised illegally) **C** lists the 10 most frequently advertised plants declared in any jurisdiction. The superscript numbers next to species names correspond to the plant photos. Photos are sourced from Getty Images and are credited to: (1) Boonsom, (2) TopPhotoImages, (3) Wjarek, (4) Igaguri_1, (5) Reginaldo Bergamo, (6) Jonnyjto, (7) ePhotocorp, (8) Radka Danailova, (9) Belizar73, (10) Membio, (11) Bdspnimage, (12) Paulfjs.

ficus-indica (Indian fig). Aquatic weed species were particularly common, including *Eichhornia crassipes* (water hyacinth) and *Limnobium laevigatum* (Amazon frogbit). *Zantedeschia aethiopica* (arum lily), an invasive geophyte, had the highest total number of advertisements for a declared plant, and the second highest number of prohibited advertisements (Fig. 5). Other frequently detected invasive plants were *Gazania* spp. (gazanias), *Hedera helix* (English ivy), *Lavandula stoechas* (topped lavender), *Rubus fruticosus* (blackberry), *Orbea variegata* (carrion flower), and *Azadirachta indica* (neem) (Fig. 5). *Limnobium laevigatum* was an example of a highly traded declared species with a far greater number of detections in jurisdictions that did not declare it. We made 19 detections for *L. laevigatum* in three prohibited jurisdictions and 69 in five permitted jurisdictions. A complete list of all declared species found and the number of prohibited and total declared detections are provided in Suppl. material 6.

We recorded the following eleven suggested uses for declared plants (Fig. 6):

1. Aquatic – filters and conditions water and provides habitat for aquatic animals (n = 72).

2. Decorative – floral arrangements, bonsai, and materials for craft projects (n = 32).

3. Groundcover – grows and covers ground well, may inhibit other plant growth or prevent erosion (n = 22).

- 4. Food edible fruits, vegetables, herbs, spices, or advertised as a superfood (n = 17).
- 5. Medicinal provides medicinal benefit (n = 11).
- 6. Screening privacy screening, hedging, or a wind break (n = 10).
- 7. Cosmetic used for cosmetic purposes such as skin care (n = 4).
- 8. Insectary attracts pollinating insects (n = 4).
- 9. Insecticide kills or repels insects (n = 3).
- 10. Air provides oxygen and purifies air (n = 2).
- 11. Spiritual incorporated into spiritual beliefs and practices (n = 1).

Sellers explicitly mentioned uses for plants in only 148 of the 1,296 advertisements of declared plants (c. 11%; 50 taxa). The most advertised use was for aquatic purposes, which encompassed actions such as improving or maintaining water quality and providing habitat for aquatic animals (n = 72). *L. laevigatum* was the declared plant most often advertised with a use, all of which were for aquatic purposes (Fig. 6). The invasive attributes of some plants interplayed with their proposed uses. For example, gazanias were advertised as groundcovers as they spread easily and form dense mats, and *Ligustrum vulgare* (privet), known for its dense vegetation, was promoted as a screening plant. A complete list of all declared species advertised with uses is provided in Suppl. material 7.

Discussion

Ornamental plant trade is the world's leading pathway for invasive plant introductions and is greatly facilitated by internet e-commerce (Humair et al. 2015; Munakamwe and Constantine 2017; Peres et al. 2018; van Kleunen et al. 2018; Beaury et al. 2021).



Figure 6. Thirteen invasive plant taxa prohibited to trade (termed declared plants) that were most frequently advertised with a use. In total, 50 declared plant taxa had uses reported in advertisements. The number of advertisements is stratified by the promoted use for the plant. These uses were reported by traders and were not verified in this study.

Our study represents the first investigation into the presence of the complete set of Australia's declared invasive plants on e-commerce. On a single popular e-commerce website, we found hundreds of opportunities to purchase a wide variety of declared plants over the course of one year. This is despite the country's strict biosecurity policies and a weed risk assessment that has been adopted by other countries (Gordon et al. 2008). Trade of invasive plants through e-commerce has been documented in other regions such as New Zealand (Derraik and Phillips 2010), the United States (US) (Maki and Galatowitsch 2004; Beaury et al. 2021) and European Union (EU) (Lenda et al. 2014; Humair et al. 2015). Australia shares similarities with the US and EU, having

accessible e-commerce platforms and easily facilitated trade across jurisdictions with differing biosecurity regulations. Our findings contribute to this growing body of evidence calling attention to e-commerce as an invasion risk pathway that is establishing globally. In particular, we have quantified the risk of illegal online plant trade conducted by individuals rather than commercial nurseries, which is a challenging aspect of e-commerce to monitor and regulate. We highlight the need to review our approaches to managing invasive species in the face of an increasingly interconnected world.

The pace of the ornamental plant trade in Australia is increasing, where 2020 saw a record high number of plant sales in the nursery industry (Horticulture Innovation Australia 2021). Given this growth and the availability of invasive plants, online trade poses a serious invasion threat and demands greater scrutiny. Since declared plant taxa have already been determined as serious biosecurity concerns (i.e., declared in State/ Territory laws), we argue that monitoring and interception of this trade is certainly warranted and should continue (Munakamwe and Constantine 2017). Low detection rates emphasise the challenge of capturing and regulating this trade. Given that our species accumulation did not approach a limit, it is likely that we have not captured the full diversity of declared plants traded online. It should also be noted that our study focussed on a narrow group of invasive plants (i.e., those that are currently declared as illegal to trade). Beyond the declared plants there are likely many other non-regulated, invasive plant species being traded on these e-commerce platforms that may still cause environmental harm (Beaury et al. 2021). Additionally, we only studied one e-commerce platform. A broader analysis of additional e-commerce platforms may reveal more declared invasive plant species that are available to the public.

In addition to the prohibited trade, declared plants were widely advertised in jurisdictions where they are currently permitted to trade. Just under half of the declared taxa and more than double the number of detections we found were located in the jurisdictions that did not prohibit sale. Some of the most frequently traded declared species are only prohibited to trade in one or two jurisdictions, despite many being known to be invasive in permitted jurisdictions. Some examples of invasive populations in permitted jurisdictions include: Lavandula stoechas in SA (Nicholson 2006), Orbea variegata in NSW (Hamilton et al. 2013), and Limnobium laevigatum in Queensland (Bickel et al. 2022). L. laevigatum was a particularly concerning example traded to a much larger extent in jurisdictions that did not prohibit its trade. We made 19 detections for L. laevigatum in prohibited jurisdictions and 69 in permitted jurisdictions. By using online trade data, we argue that jurisdictions should reconsider the risk of invasive species like L. laevigatum to determine if prohibition is warranted. A similar situation has been observed in the US (Beaury et al. 2021), another geographically large country with multiple states with their own governing legislations. Like in the US, we argue this type of trade can compromise the biosecurity of neighbouring jurisdictions (Beaury et al. 2021). For example, we found NSW and Vic. traded large quantities of species declared in neighbouring jurisdictions. This is especially concerning because the plant trade facilitates long-distance dispersal from plants mailed over long distances (Maki and Galatowitsch 2004). Despite the limitations of online trade data, it is still a valuable resource to help identify species or areas of concern (Kikillus et al. 2012). Thus, we suggest future weed risk assessments utilise data collected from monitoring e-commerce to factor in trade of invasive plants as a risk factor, even if occurring in other jurisdictions. Incorporating this may lead jurisdictions to consider a nationally consistent approach to plant declarations, similar to other control programs which have benefited from cross-border coordination (Pluess et al. 2012). As long as the trade of invasive plants persists somewhere with a country, the risk of natural or human mediated dispersal into vulnerable landscapes will remain.

While more consistent regulations among jurisdictions would provide the legal framework to address invasive plant trade, our results may suggest this is not a cureall. We found that across declared plant taxa, there was no difference in the quantities of advertisements observed in prohibited and permitted jurisdictions. We also saw no significant effect on price, however our sample size was reduced to 20 declared taxa, making it difficult to draw a meaningful conclusion across all declared taxa traded. It is likely that jurisdictional regulations are reducing the total abundance of declared taxa in Australian plant trade, through compliance from traditional "brick-and-mortar" nurseries. It is important to note that the lack of effect on quantity we saw could be due to the limited size of our sample. Investigations across larger datasets, and across more e-commerce platforms, may reveal different results. However, if trade prohibition is not having an effect on the quantity of online trade, explanations from other plant trade studies may provide an answer. For one, sellers may perceive online trading of declared plants as low risk. This perception may be in part due to limited enforcement of e-commerce due to surveillance and legal challenges (Lavorgna and Sajeva 2021; Whitehead et al. 2021). Another reason may be a lack of awareness that these plants are invasive and that their trade is prohibited. Public awareness has been suggested by other studies into invasive plant trade, reporting that people are often unaware, lack the ability to correctly identify plants, or are misinformed about relevant legislation rather than knowingly breaking the law (Derraik and Phillips 2010; Martin and Coetzee 2011; Munakamwe and Constantine 2017). We suggest implementing web scraping surveillance tools to improve enforcement and to enhance public knowledge through awareness campaigns which improve invasive species management (Novoa et al. 2017; Cordeiro et al. 2020; Li et al. 2021). Further, e-commerce platforms can also play a role in prevention and should be engaged as a biosecurity stakeholder. Specifically, in agreement with other studies of the illegal plant trade, we recommend that relevant governments coordinate with e-commerce websites to prevent illegal trade (Derraik and Phillips 2010; Munakamwe and Constantine 2017). For example, e-commerce websites could provide information to people creating plant advertisements, warning them of plants that cannot be sold and to help identify those plants.

Given that plant trade is fundamentally human driven, we expected to observe a higher number of advertisements matching search terms and corresponding to declared plants in jurisdictions with larger populations. Consequently, in the targeted sample, we observed this trend with NSW and Vic. having the greatest number of total declared advertisements. Interestingly, NSW and Vic. also had the greatest proportion of total declared advertisements. However, in terms of prohibited advertisements, WA, SA, and NT had the highest proportions in the targeted sample. To explain this, we should consider the plants that jurisdictions have chosen to declare. Regulations are jurisdiction based, therefore differences in declarations arise between jurisdictions. WA declares the greatest number of plant taxa of any Australia jurisdiction (877 plant taxa), more than double that of the next highest jurisdiction. As a result, WA prohibits a larger proportion of Australia's assemblage of declared plants. Complementary to this is that NT, SA, and WA declare highly traded declared species that other jurisdictions do not. *Zantedeschia aethiopica* is only declared in SA and WA, *Opuntia ficus-indica* is only declared in NT and WA, and *Gazania* spp. are only declared in SA. These species were frequently traded in SA and WA, thus the higher proportions are indicative of the regulations of these jurisdictions. However, NT prohibited advertisements were predominately for aquatic declared plants that are not exclusively declared in the jurisdiction. Evidently this is a popular group of plants traded in the jurisdiction, one that may benefit from targeted management campaigns.

We found that Opuntia cacti and aquatic invasive plants were among the most frequently traded declared plants. This is concerning given the historical extent of Opuntia impact on the Australian environment (Freeman 1992), and the invasiveness of the traded aquatic weeds Eichhornia crassipes and L. laevigatum (Riches 2001; Tidwell and O'Donnell 2010; Villamagna and Murphy 2010). It is possible that some traits that aid their invasion success could also lend to their popularity in trade. Opuntia cacti are easily propagated from cuttings and will do so readily when discarded from gardens (Smith 2006; Smith et al. 2011). E. crassipes and L. laevigatum can also reproduce vegetatively and in good conditions growers will quickly have an overabundance (Madsen and Morgan 2021; Prasetyo et al. 2021). This ease of excess could present sale as an attractive option to get rid of surplus plants, thus facilitating invasions. However, without further investigation into seller behaviour we cannot say how common this is. Similarly, it has been suggested that some Opuntia protective traits (e.g., spines and glochids) eventually lead owners to dispose of them. Smith et al. (2011) suggested that the irritating hairs (glochids) of *Opuntia microdasys* drive owners to dispose of the plants through dumping. We spoke with a compliance officer investigating Opuntia sales, who reported that sellers mention a desire to sell the plants in order to be rid of them (D. Swan 2021, pers. comm., 3 November). The high number of advertisements we observed of these taxa may indicate selling plants is an attractive alternative to disposal but this would require further investigation.

We demonstrated that targeted searches using string matching was a more effective means of detection than random sampling. We took a conservative approach by including common and generic names (e.g., pond plant) alongside scientific names in our effort to detect declared plants. Common and generic names are non-specific and can be shared by many plant species, contributing to a higher rate of false positives. However, we believe this approach is necessary to reduce the chance of missing advertisements for invasive species. Image recognition technology could be employed to further increase detection rate (Di Minin et al. 2019). However, the accuracy of image recognition is dependent on large, pre-identified image datasets and the quality of images provided (Xiong et al. 2021). The quality of images that we observed in advertisements varied greatly in resolution and often had complex backgrounds, a feature known to hinder the accuracy of image recognition (Xiong et al. 2021). We propose that string matching and other natural language processing methods are a cost-effective means for the semi-automated detection of invasive plants on e-commerce platforms.

The advertised uses for declared plants revealed some reasons why people desire them, which may complicate their management. We discovered a variety of uses advertised for declared plants, including food, medicine, cosmetics, and decoration (e.g., floral arrangements). However, the most commonly advertised uses fell into the 'aquatic' category, uses such as water-conditioning and providing habitat for aquatic pets. Perceived water-conditioning abilities could encourage people to introduce the plant into waterbodies (e.g., ponds and dams), risking dispersal into the surrounding environment. For example, we found E. crassipes traded which has been known to be intentionally introduced into waterbodies to help prevent algal blooms (Villamagna and Murphy 2010). It is important to consider people's intended use of an invasive plant because prevention is often more of a cultural challenge than biological (Pfeiffer and Voeks 2008). Understanding the public's desire for an invasive plant could help to tailor education campaigns or promote non-invasive alternatives. It is also important that public attitudes are understood to establish collaborative efforts between invested communities and policymakers, which will lead to optimal social and biosecurity outcomes (Virtue et al. 2004; Head 2017).

Conclusion

We observed the prohibited advertisement of invasive plants online in all Australian jurisdictions. This online trade creates many opportunities for the public to purchase and spread declared invasive plants around the country. As it stands, laws prohibiting the trade of declared plants have not halted prohibited advertisements of declared plants on public e-commerce. We suggest enhancing detection methods of illegal trade using web scraping techniques to improve enforcement. Jurisdictions should also focus on educating the public that certain plants are prohibited to trade while considering the desire that people have for these plants to help promote safe alternatives. Cooperation should be sought from e-commerce websites to prevent instances of illegal trade being facilitated on their platforms. For now, monitoring e-commerce is still needed and we have demonstrated that web-scraping is an effective tool. Data collected from monitoring e-commerce could also be utilised in future weed risk assessments with online availability incorporated as a risk factor. Beyond surveillance, jurisdictions should seek to better align the taxa they choose to regulate as the existing legal disparities could contribute to the persistence of invasive species being distributed within a country. Australia's biosecurity, and that of other countries and regions, would benefit from more coordinated approaches to controlling the online trade of invasive species.

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Supplementary material I

This table details the relevant legislation identifying declared plants in each jurisdiction Author: Jacob Maher

Data type: Legislation references

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl1

Supplementary material 2

Short-list of invasive plants used for surveying candidate Australian websites Author: Jacob Maher

Data type: species list (PDF file)

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl2

Supplementary material 3

List of search term exceptions used to remove the majority of false positives in target sample dataset

Author: Jacob Maher

Data type: Search term exceptions (PDF file)

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl3

Supplementary material 4

The price of plants advertised online in Australia from a random sample of 625 advertisements from each jurisdiction

Author: Jacob Maher

Data type: Boxplot (PDF file)

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl4

Supplementary material 5

Distribution of the mean difference in price for declared plant taxa between prohibited and permitted jurisdictions

Author: Jacob Maher

Data type: image (PDF file)

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl5

Supplementary material 6

Total number of detections for invasive plants which are prohibited to trade in at least one Australian jurisdictions

Author: Jacob Maher

Data type: Table: Species detections (PDF file)

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl6

Supplementary material 7

The number of observations for plant taxa prohibited to trade (i.e., declared plants) that were advertised with uses by traders

Author: Jacob Maher

Data type: table: Species detections with uses (PDF file)

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Link: https://doi.org/10.3897/neobiota.87.104472.suppl7
RESEARCH ARTICLE



Stable isotope values and trophic analysis of invasive three-spined stickleback in Upper Lake Constance points to significant piscivory

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Abstract

The three-spined stickleback *Gasterosteus aculeatus* was introduced into Lake Constance in the 1940s and occupied a limited range until late 2012. Since then the species has expanded from a solely littoral habitat in Upper Lake Constance, but now makes seasonal migrations into the pelagic zone. This behavioral change has been accompanied by a drastic increase in stickleback abundance. In order to integrate information about feeding of sticklebacks in Upper Lake Constance over two consecutive years, stomach content analysis was combined with seasonal stable isotope analysis on two types of tissue (muscle and liver). Isotope values were also obtained for zooplankton, whitefish larvae and eggs. We calculated the contribution of potential food sources for sticklebacks' diet using a Bayesian mixing model (SIMMR). Furthermore, we determined stickleback trophic position, and δ^{15} N and δ^{13} C values were compared with those of other fish species of Lake Constance. The results of the Bayesian model as well as the stomach content analysis showed clear evidence of stickleback predation on fish eggs and larvae. Stickleback δ^{15} N values were reduced, and those of whitefish larvae were similar to those of sticklebacks after accounting trophic fractionation of N isotopes. Trophic position calculations further identified sticklebacks as piscivorous,

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while the δ^{13} C values of the liver and stomach content analysis suggests that a benthic-pelagic species pair may exist in Lake Constance. These findings support the hypotheses that sticklebacks in Lake Constance can display piscivorous feeding behaviour on sympatric fish species, most likely whitefish larvae and eggs.

Keywords

Carbon littoral source, Gasterosteus aculeatus, larvae predation, niche overlap, trophic position, whitefish

Introduction

Aquatic invasive species (AIS) are of concern worldwide due to their devastating impacts on ecosystems and economies (Havel et al. 2015; Treanor et al. 2017). Furthermore, the number of AIS in freshwater ecosystems will increase in the future because AIS are still translocating outside of their native ranges and in many cases AIS have not fully occupied their potential ranges (Strayer 2010a). Additionally, the introduction of AIS drives changes in the structure and functioning of existing ecosystems and complicates their management (Strayer 2010b). Therefore, knowing the trophic niches and consumption impacts of AIS is crucial to addressing the implications for food webs and ecosystems (Bodey et al. 2011). This is especially true in Lake Constance, one of the largest lakes in Central Europe, where the three-spined stickleback (Gasterosteus aculeatus L., 1758), hereafter referred to as stickleback, was introduced accidentally in the mid-1940s (Roch et al. 2018), because it is known that sticklebacks could have detrimental effects on native fish community if hyperabundant stickleback population established (Bergström et al. 2015; Byström et al. 2015). For over half a century the species existed almost exclusively in small swarms near the shore but, since the end of 2012, has expanded from littoral habitats into the pelagic zone of Upper Lake Constance (ULC) and undergone a drastic increase in abundance (Eckmann and Engesser 2019; Gugele et al. 2020). Only two years later, in September 2014, stickleback shoals represented 96% of all pelagic fish and 28% of the total pelagic fish biomass (Eckmann and Engesser 2019), a globally unique situation for a large and deep oligotrophic lake. After 2014, average stickleback abundances plateaued to between 1280 and 7990 individuals/ha in the pelagic zone of Lake Constance (Eckmann and Engesser 2019), with a peak abundance of 19100 individuals/ha recorded in September 2017 (Gugele et al. 2020). Recent stomach content analysis of sticklebacks has identified zooplankton (Daphnia spp.) as their main prey (Ogorelec et al. 2022). Since the native pelagic whitefish (Coregonus wartmanni Bloch, 1784) also mainly feed on zooplankton (Eckmann and Rösch 1998; Ogorelec et al. 2022), high stickleback abundances may have deleterious impacts on native whitefish via interspecific competition. By 2015 the yield of native whitefish, the main target species of the local fishery (Baer et al. 2017), had declined drastically from around 300-600 mt (metric tons) before stickleback invasion to less than 150 mt (Roch et al. 2018). At the same time, the average weight of whitefish (age classes 3–5) captured in the pelagic zone has undergone a 17%–51% decline (arithmetic mean: 33%) (Rösch et al. 2018). Besides, integrated bioenergetics

and growth modelling revealed increasing competition with sticklebacks as a chief factor in reduced growth of whitefish in the lake (DeWeber et al. 2022).

There is also evidence that sticklebacks in Lake Constance may impact whitefish as predators of whitefish larvae and eggs (Roch et al. 2018; Rösch et al. 2018; Ros et al. 2019; Gugele et al. 2020; Baer et al. 2021). Whitefish spawn in November and December (Eckmann 1991) and whitefish larvae with a total length below 4 cm, which resembles the gape limitation of sticklebacks (cf. Roch et al. 2018) can be found in Lake Constance from February until the end of May (Eckmann 1989). Additionally, stickleback aggregations were documented in habitats where the newly hatched whitefish larvae were found from March to April, both in the pelagic zone and near the shoreline (Gugele et al. 2020). However, it should be kept in mind that pelagic whitefish spawn near the water surface in the open waters of the lake (Eckmann 1991) and their eggs sink relatively quickly to depths as great as 250 m (Eckmann and Rösch 1998), leaving a short time window to predate on them. Nevertheless, sticklebacks are able to locate whitefish eggs even during complete darkness (Baer et al. 2021) but only forage in depths down to around 30 m (Eckmann and Engesser 2019; Gugele et al. 2020), so any consumption of whitefish eggs must take place before they sink and become unattainable.

Furthermore, the distribution of the whitefish larvae is normally patchy (Ransom et al. 2022) and evidence of predation of whitefish larvae by sticklebacks may be only coincidentally available for single sticklebacks. These factors mean the role of sticklebacks in Lake Constance food web, including their impact on native lake whitefish, is not entirely simple, and predation of whitefish eggs and larvae may be a rare behaviour, posing only a minor threat to naturally hatched or stocked whitefish larvae. However, each year 100–200 million larvae between 18–25 mm in size are stocked in the littoral and pelagic zone across the whole lake (Baer et al. 2023). Consequently, it is hypothesized that the stocked larvae may act as an easily available, nutrient and energy rich food source in the oligotrophic water that might facilitate the stickleback invasion's development, permitting the species to fully exploit the pelagic habitat while simultaneously reducing recruitment of whitefish (Roch et al. 2018).

Therefore, to get more insight into the feeding ecology of sticklebacks in ULC, we performed monthly stable isotope analysis of stickleback muscle and liver tissue over a two-year period. In addition to providing information on the diet of an organism over time, stable isotope analysis can illuminate feeding habitats, quantify complex interactions, and be used to track elements, energy, or mass through food webs and ecosystems (Post 2002; Janjua and Gerdeaux 2011). Stable isotope ratios of carbon and nitrogen have been applied extensively in food web studies over the past 30 years (Janjua and Gerdeaux 2011) and have proved useful in assessing the impacts of invasive species on trophic structures (Vander Zanden and Rasmussen 1999; Bodey et al. 2011). In the present study, stable isotope analysis was carried out on stickleback and their potential prey in the pelagic zone (zooplankton, whitefish eggs and larvae) alongside classic stomach content analysis and stable isotope mixing models to address the following four research questions: 1) Do the δ^{15} N values and trophic position of sticklebacks reflect the seasonal feeding of whitefish larvae and eggs or that of other

food sources, such as zooplankton? 2) Where do invasive sticklebacks sit relative to other fish species in the trophic structure of Lake Constance? 3) How does the trophic position of sticklebacks in Lake Constance compare to that of conspecifics in similar ecosystems? 4) Are there any differences in isotopic signature between sticklebacks caught in littoral and pelagic habitats? The answers to these questions have implications beyond Lake Constance because similar tendencies, i.e. increased predation by introduced sticklebacks on native fish species, are also being observed elsewhere (Ljunggren et al. 2010; Bergström et al. 2015; Byström et al. 2015).

Materials and methods

Ethics statement

Approval of the present study by a review board institution or ethics committee was not necessary because all fish were caught under permits issued by the local fisheries administration (Regierungspräsidium Tübingen), by qualified (license-holding) personnel subject to regular checks by the local fisheries administration (Regierungspräsidium Tübingen). All fish were caught according to the German Animal Protection Law (Tierschutzgesetz § 4) and the ordinance on slaughter and killing of animals (Tierschutzschlachtverordnung § 13).

Sampling of sticklebacks

Lake Constance is part of the Rhine drainage basin and is bordered by Austria, Germany and Switzerland (47°38'N, 9°22'E). The total surface area of 536 km² is divided between the large (472 km²), deep (>250 m) Upper Lake (ULC) and the smaller (63 km²), shallower Lower Lake (LLC). Due to missing data and lack of knowledge about the stickleback situation in LLC and different type of lake (warm, mesotrophic), this basin was excluded in the present study. Thus, the current study only focusses on warm monomictic, large oligotrophic pre-alpine basin of ULC. The fish community of ULC comprises a minimum of 30 species (Eckmann and Rösch 1998) of which about 10 are targeted by professional fishermen (Rösch 2014). Of these, whitefish (*Coregonus* spp.) are the most economically important, and fisheries management is based on routine monitoring of this important group (www.ibkf.org). An overview of the fisheries situation is given by Baer et al. (2017).

Stickleback sampling of ULC was conducted monthly, from March 2017 until November 2018, using littoral and pelagic gillnets with mesh sizes of 10–12 mm. All nets had a height of 3 m, while length varied with mesh size: 30 m for nets with 10 mm mesh and 15 m for the 12-mm mesh net. All pelagic nets were deployed to drift freely behind the nets used in the monthly monitoring of whitefish (mesh sizes 36–44 mm), at depths of 3–15 m according to the areas of greatest stickleback abundance recorded during hydroacoustic surveys (Gugele et al. 2020). Benthic nets were set at depths from 6 to 20 m. All nets were set overnight, with a soak time of about 15 h. The overall

catch in the pelagic gillnets (number per unit effort, NPUE, as n/m^2 per net) was low from January to September (NPUE 0.03–0.25) and peaked between November and December (NPUE 1.1–7.7), the spawning season of whitefish (Baer et al. 2022b). Catches in the benthic gillnets were highest during the stickleback spawning season between May and July (NPUE 11.8–59.0) and a second peak was again observed during November and December (NPUE 6.0–79.0; Baer et al. 2022b).

10 to 34 samples of stickleback white muscle and liver tissue were taken from each monthly catch. Catches of fewer than 10 individuals (recorded in August and September each year, plus April 2017, July 2017 and October 2018) were excluded from analysis. C and N stable isotope analysis was run on 275 sticklebacks. Of these, 193 were caught in the littoral zone and 82 in the pelagic zone. All fish were euthanised with an overdose of clove oil (1 mL L⁻¹) and a gill cut. They were measured post-mortem (total length (TL) to the nearest mm), weighed to the nearest 0.01 mg and sex was recorded. Some sticklebacks were infested with the pseudophyllidean cestode *Schistocephalus solidus*, and because it is known that the health status of a fish can have direct effects on the stable isotope values (Karlson et al. 2018), a combined parasite-to-host biomass ratio (parasite index, *PI*) was calculated as an indirect measure of the severity of infestation (Baer et al. 2022b). All parasites were counted per host, blotted, and weighed to the nearest 0.01 mg and *PI* was determined using the formula

$$PI = P/H \tag{1}$$

where P is the total weight of the parasites and H is the mass of the host without the parasite.

Due to internal procedures, gastrointestinal tracts (stomach and intestine) were analysed from a subsample of 109 sticklebacks; 69 caught in the pelagic zone and 40 caught in the littoral zone (TL 68 mm \pm 6 mm standard deviation SD). Samples were taken during all four seasons (autumn 2017, winter 2017, spring 2018, and summer 2018), and for each season and each habitat, the gut contents of at least 10 individuals were analysed with the exception of some sampling dates (20 during winter and summer in both pelagic and littoral zone, and 19 during spring in the pelagic zone). Food items were identified and counted in a zooplankton counting chamber and categorised into five groups, namely copepods (nauplii, copepodites and copepods of Cyclopoida, Calanoida and Harpacticoida); Bosmina (all members of the genus Bosmina); other herbivorous/detritivorous cladocerans (Daphnia spp., Diaphanosoma brachyurum and Chydoridae); predatory cladocerans (Bythotrephes longimanus and Leptodora kindtii); fish (eggs and larvae) and other (Chironomidae, Annelida, Bivalvia, Collembola, Ceratopogonidae, Ephemeroptera, adult Heteroptera, Hydrachnidia, adult Mysidae, Nematoda, Ostracoda, Plecoptera, Simuliidae and Trichoptera). Diet quantification of sticklebacks followed the use of the numerical method and diet was calculated as a percentage of the total number of prey items eaten per stickleback (Amundsen and Sánchez-Hernández 2019). Furthermore, we calculated for each season and habitat the mean number (\pm standard deviation SD) of consumed food items per category.

Stable isotope analysis of sticklebacks, the diet of sticklebacks and other fish species

Although stable isotope analyses on fish C and N are generally performed using muscle tissue alone, the more rapid turnover of liver tissue means isotope signatures there reflect more recent feeding (Boecklen et al. 2011). Repeated at suitable intervals, in this case monthly, these tissue-based differences in signal lag can help resolve the timing of hard-to-predict peaks in seasonal prey availability, such as that of whitefish eggs and larvae.

Tissue samples from sticklebacks caught in 2017–18 were prepared for analysis by drying them in an oven at around 60 °C for 48 hr and grinding them into a fine powder. Lipid extraction was performed on the samples because some studies have shown that in tissues with C:N ratios greater than 3.5, such treatment reduces bias in δ^{13} C values (Skinner et al. 2016). Therefore, lipid extraction of samples was conducted by adding 200 µL of 2:1 Chloroform:Methanol mixture to the powdered tissue. Afterwards, samples were vortexed and centrifuged for two minutes at 4000 rpm. The excess sample was discarded, and centrifugation was repeated 2–4 times until the sample colour changed from yellow to colourless. Samples were then washed in 200 µL Milli-Q water, followed by further vortexing and centrifugation for 2 min (4000 rpm). Again, the excess sample was discarded, and washing was repeated multiple times during the lipid extraction. Next, samples were dried in a fume hood for 48 hr, then ground again to a fine powder and weighed (ca. 0.7 mg) to the nearest 0.001 mg in tin capsules, using a microbalance (Chyo Balance Corporation, Kyoto, Japan).

To measure isotopic ratios samples were combusted in a vario MICRO cube elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany). The emerging gases were separated via gas chromatography and passed into a Micromass Isoprime isotope mass spectrometer (Isoprime Ltd., Cheadle Hulme, UK) for determination of the 13C/12C and 15N/14N ratios (R). Measurements are reported in δ -notation (δ^{13} C, δ^{15} N) in parts per thousand deviations (‰), where $\delta = 1000 \times (R_{sample}/R_{standard} - 1)$ relative to the Pee Dee Belemnite (PDB) for carbon and atmospheric N₂ for nitrogen. Two sulphanilamide (Isoprime internal standards) and two casein samples were used as laboratory standards for every 10 unknowns in the sequence. Replicate assays of internal laboratory standards indicated measurement errors (SD) of $\pm 0.05\%$ and 0.15% for δ^{13} C and δ^{15} N, respectively.

To compare the isotopic values of sticklebacks with those of other species of fish in ULC, 128 additional sampled of muscle tissue were analysed from bleak (*Alburnus alburnus* L., 1758; n = 18; mean TL 60 mm \pm 6 mm SD) roach (*Rutilus rutilus* L., 1758; n = 34; mean TL 263 mm \pm 71 mm SD); rudd (*Scardinius erythrophthalmus* L., 1758; n = 12; mean TL 217 mm \pm 40 mm SD); tench (*Tinca tinca* L., 1758; n = 19; mean TL 226 mm \pm 147 mm SD); pelagic whitefish (n = 21; mean TL 308 mm \pm 55 mm SD); burbot (*Lota lota* L., 1758; n = 19; mean TL 374 mm \pm 38 mm SD); and pike (*Esox lucius* L., 1758; n = 5; mean TL 289 mm \pm 44 mm SD). All were sampled with gill nets during August and September 2020. All fish were euthanised with an overdose of clove oil (1 mL L⁻¹) and a gill cut. Using data from stomach content analyses carried

out in prior studies, all fish species were divided into different feeding guilds: whitefish were categorised as zooplanktivorous; burbot as partly piscivorous (Hansen et al. 2022); pike as piscivorous; roach, bleak, and tench as benthivorous/insectivorous; and rudd as herbivorous (Baer et al. 2022a). Due to the stomach content analysis from this study, sticklebacks were categorised as omnivorous. Samples of white muscle were excised and frozen (-20 °C) until further processing.

For calculating the trophic position of sticklebacks, pike and burbot, faucet snails (*Bithynia tentaculata*, n = 10) were collected in August 2020 from the littoral habitat and used for the estimation of the littoral baseline ($=\delta^{15}N_{lit.base}$). Quagga mussels (*Dreissena rostriformis bugensis*, n = 200) were collected from free-standing piles in the pelagic zone 0.5–2 m depth in the upper mixed layer of Lake Constance and used for the estimation of the pelagic baseline ($=\delta^{15}N_{pel.base}$).

To gain more insight into the isotopic signatures of potential stickleback prey during winter and spring, five samples of zooplankton (wet weight (g): mean = 2.34, SD = 2.53) were netted with 300 μ m mesh in the epilimnion of ULC, first in October and December 2021, then in February, March, and early May 2022. An abundance of pollen in the lake epilimnion during April 2022 prevented an uncontaminated sample being taken during that month. In addition, in December 2021, 36 females of C. wartmanni (pelagic whitefish) and 42 C. macrophthalmus (benthic whitefish) were caught during spawning at their spawning grounds in ULC as part of routine sampling conducted by the Fisheries Research Station of Baden-Württemberg. To get the isotopic signature of whitefish eggs and larvae, a small sample of eggs was taken from each individual and larvae hatched from the eggs of pelagic whitefish (kept at a hatchery facility in Langenargen, Baden-Württemberg) were also sampled. After hatching, larvae were held in rearing vats until the yolk sac was partly absorbed and larvae had begun to exhibit normal swimming behaviour. From these non-fed, free-swimming larvae, four subsamples of multiple individuals (n: mean = 172, SD = 100) were taken and euthanised with an overdose of carbonated water. Clove oil was avoided in this instance as it may have biased isotopic readings, and unlike larger fish, the delicate larvae cannot be easily washed without damage. To remove potential biases due to the length of time between the main stickleback sampling (2017–2018) and the sampling of the zooplankton, whitefish eggs and larvae (2021–2022) (Fig. 1), a further 50 sticklebacks were caught for additional stable isotope analysis in January, February and April 2022 (25 each in the pelagic and littoral zone, in total 150) using gill nets.

Samples of sticklebacks caught in 2022 and other fish were prepared for stable isotope analysis via freeze drying at -50 °C under pressurisation (<1 mbar), and ground to homogenous powder using a mixer mill. Whitefish egg samples were dried at 60 °C in a drying oven, before being stored in a glass desiccator filled with silica desiccator beads; the desiccator was stored in a cool, dark environment. Samples of plankton and pelagic whitefish larvae were dried overnight in a drying oven (60 °C), then stored in freezers at -20 °C. Each individual dried sample of plankton, whitefish larvae and whitefish eggs were then separately homogenised using a BeadRupture Homogenizer (Omni International, Kennesaw, Georgia, United States) by dispensing the sample



Figure 1. Timeline of sampling in the present study.

into a plastic microtube, with a number of sterilised metal beads (<0.5 mL), and processing the sample into a fine, homogenous powder. The times and speeds used in the homogenisation process were adapted according to the individual condition of the samples. After homogenisation, samples were stored in freezers at -20 °C. Sample powder (0.3-0.4 mg) was weighed into tin capsules and combusted in an isotope ratio mass spectrometer (Delta plus, Finnigan MAT, Mas-Com GmbH, Bremen, Germany), interfaced (viaConFlo II, Finnigan MAT, MasCom GmbH, Bremen, Germany) with an elemental analyser (EA 1108, CarloErba, Thermo Fisher SCIENTIFIC, Milan, Italy). Because the mean C:N values $(\pm SD)$ of all fish samples were below 3.5, lipid extraction of fish muscle tissue was not conducted (Matthews et al. 2010; Skinner et al. 2016). Measurements are reported in δ -notation (δ^{13} C, δ^{15} N) in parts per thousand deviations (‰), where $\delta = 1000 \times (Rsample/Rstandard - 1)$ relative to the Pee Dee Belemnite (PDB) standard for carbon and atmospheric N₂ for nitrogen. Finely ground animal horn (keratin) was used as a laboratory standard for every 10 unknowns in sequence. Replicate assays of internal laboratory standards indicated measurement errors (SD) of \pm 0.05% and 0.15% for δ^{13} C and δ^{15} N, respectively.

Trophic position of sticklebacks

The trophic position of sticklebacks was calculated according to the protocol established by Post (2002) for a two-source food web:

Tophic position =
$$\lambda_{\text{Base}} + (\delta^{15}N_{\text{stickleback}} - [\delta^{15}N_{\text{lit. base}} * \alpha + \delta^{15}N_{\text{pel. base}} * (1 - \alpha)])/\Delta_n$$
 (2)

$$\alpha = \frac{\delta^{13}C_{\text{stickleback}} - \delta^{13}C_{\text{pel,base}}}{\delta^{13}C_{\text{lit,base}} - \delta^{13}C_{\text{pel,base}}}$$
(3)

where λ_{Base} denotes the trophic position of the consumer ($\lambda_{Base} = 2$) used for the estimation of the littoral (= $\delta^{15}N_{lit, base}$) and pelagic (= $\delta^{15}N_{pel, base}$) baseline. The isotope values of faucet snails and quagga mussels were used for $\delta^{15}N_{pel, base}$ and $\delta^{13}C_{pel, base}$. As filter feeders, quagga mussels are an ideal integrator species for representing the consumer base of the pelagic food web, and are favoured over bulk seston or plankton samples, which may include non-consumer material and undifferentiated detritus and thus bias stable isotope ratio signatures. The isotopic ratios of quagga mussels and faucet snails were assessed using the same method applied for plankton, whitefish eggs and larvae (see

above). $\delta^{13}C_{stickleback}$ is the measured $\delta^{13}C$ value of sticklebacks muscle. $\delta^{15}N_{stickleback}$ is the measured $\delta^{15}N$ value of sticklebacks muscle, Δ_n is the enrichment in $\delta^{15}N$ per trophic level ($\Delta_n = 3.4$ (Minagawa and Wada 1984; Vander Zanden and Rasmussen 1999; Post 2002; Reimchen et al. 2008)) and α denotes the proportion of sticklebacks carbon derived ultimately from littoral sources. A Δ_n value of 3.4 is commonly used for sticklebacks (Post 2002; Matthews et al. 2010), but in the light of recent reviews suggesting a lower level of $\delta^{15}N$ enrichment for carnivorous fish (Vanderklift and Ponsard 2003; Boecklen et al. 2011; Blanke et al. 2017; Kambikambi et al. 2019), a further analysis as included using $\Delta_n = 2.0$ as a value for the trophic enrichment of $\delta^{15}N$ in sticklebacks.

To compare the trophic position of sticklebacks with piscivorous fish species, the trophic position for pike and burbot was then calculated for each pike and burbot using the formula: Trophic position = [(piscivorous fish $\delta^{15}N - \delta^{15}N_{lit. base})/\Delta_n] + \lambda_{Base}$ (Nyqvist et al. 2018).

Statistical analysis

To test the effects of covariates on the $\delta^{15}N$ or $\delta^{13}C$ of muscle or liver tissue and the trophic position of sticklebacks, the following general linear model (GLM) (Sachs 1997) was used:

$$Y_{ijklmno} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \delta_l + \varepsilon_m + \zeta_n + \eta_o + \theta_{ijklmno}$$
(4)

where $Y_{ijklmno}$ is $\delta^{15}N$ or $\delta^{13}C$ in muscle or liver tissue or the trophic position of sticklebacks; μ is the overall mean, α_i denotes month, β_j is total length, $(\alpha\beta)_{ij}$ is the interaction between month and total length, γ_k represents year and was added to the model as a random factor, δ_l is habitat (pelagic or littoral zone), ε_m is sex (male or female), ζ_n denotes the infection state (yes/no), η_o is parasite index and $\theta_{ijklmno}$ is the random residual error. Model requirements, *i.e.* residuals not violating linearity, normality or non-independence were checked by inspecting residuals (predicted vs. expected plots) and multicollinearity by inspection correlation of independent variables. Single outliers with extreme values were excluded from the dataset (selection criteria: more than eight times standard deviation). Student's t-test was used for *post hoc* comparisons between habitat and sex after testing for homoscedasticity (Levene test) and by building contrasts (Sokal and Rohlf 2003). The GLM for trophic position of sticklebacks was also run with Δ_n set at 2.0.

Differences in mean δ^{15} N and δ^{13} C values among Lake Constance fish species were examined using Tukey-Kramer HSD-tests.

The contribution rate of potential food sources for sticklebacks' diet was estimated using the Bayesian mixing model in the SIMMR package (Parnell and Inger 2019), which was based on the SIAR package (Parnell et al. 2010), and implemented in the R 4.04 software (R Core Team 2020). We run separated mixing models for isotopic signatures of the liver samples of sticklebacks collected in winter (December to March) and in the summer (June and July). We choose to use only liver values because isotope values of the liver react faster than those of the muscles (time lag of only one month, Perga and Gerdeaux 2006). We include as potential food sources the isotopic values for whitefish larvae and eggs of both whitefish species, plankton, burbot, pike, and chironomids from both pelagic and littoral habitats. Whitefish eggs and larvae are only available for stickleback during winter (Roch et al. 2018; Baer et al. 2021); as such, they were not included in the model for summer samples. Furthermore, we used the isotopic values from burbot and pike as a proxy for the food source "fish", because eggs and larvae of pike are known to be a food source for sticklebacks (Bergström et al. 2015) and the larvae of burbot are small and are available for sticklebacks the whole year round (Probst and Eckmann 2009). The δ^{15} N and δ^{13} C values of chironomids from both habitats were taken from previous samplings in Upper Lake Constance, made in 2015-2017, representing mean yearly values (kindly provided by M. Sabel and D. Straile). As correction factors, we used the widely accepted trophic enrichment factors (TEFs) (Cui et al. 2021) to estimate the direct contribution of food sources to detritivores (chironomids) and planktivores (whitefish) and omnivores (stickleback), and the indirect contribution of food sources to sticklebacks caught in the pelagic and littoral zone during winter and summer. In order to simplify the model and to enhance interpretability, we performed a-posteriori combination of some of the food sources that fell on similar regions of the isospace plot (Suppl. material 1: fig. S1). We combined the isotopic values of both species of whitefish eggs and larvae to category "Whitefish eggs" and "Whitefish larvae", the chironomids from both habitats to "Chironomids", and the burbot and pike to new category "Fish". Furthermore, to get more insight into the importance of the prey category "Fish", we also ran the model without this category. SIMMR relies on a Markov Chain Monte Carlo to find possible solutions and disregards those not probabilistically consistent with the data. The iterations run were 10^4 , the burn-in was 10^3 , the posterior was thinned by 10, and the number of chains fit was 4.

Furthermore, data from all examined fish species were pooled according to feeding guild in order to calculate a standard ellipse area corrected for small samples (SEA₂). The SEA_c represents the core isotopic niche of each guild after factoring in maximum likelihoods. It comprises around 40% of data and resembles a two-dimensional measurement of standard deviation (Jackson et al. 2011). The small sample size-corrected standard ellipse area (SEA_c) represents the core isotopic niche area of the individuals sampled. The isotopic niche overlap of omnivorous sticklebacks with the remaining feeding guilds was subsequently calculated as a proportion of the sum of non-overlapping areas of the SEA_cs. All analyses were performed using the SIBER package (Stable Isotope Bayesian Ellipses in R, v. 2.15; (Jackson et al. 2011)) in R (v. 4.04, (R Core Team 2020)).

To compare the trophic position of sticklebacks in Lake Constance to that of conspecifics in similar ecosystems (to see if the position in ULC is common), the trophic positions of sticklebacks from Lake Constance (here: mean value of all sticklebacks, independent of habitat) were compared to lake populations from North America (Matthews et al. 2010) and Norway (Østbye et al. 2016) using a z-test on arithmetic means and standard deviation with *post hoc* Bonferroni correction. We used for this comparison all lakes cited by Matthews et al. (2010) and only lake populations from Norway with a clear assignment to the isotopic values of sticklebacks (Einletvatn and Farstadtvatn lake). The same trophic position calculation (according to Post 2002) and the same trophic enrichment factor ($\Delta_p = 3.4$) used in this study also applied to analyses of these lake systems.

Unless further specified, all statistics were performed in JMP Pro 15.1 (64 bit, SAS Institute).

Results

Stable isotope values of sticklebacks (main sampling)

For sticklebacks sampled between 2017 and 2018 (mean TL 66 mm \pm 5 mm (\pm SD)), the mean δ^{15} N value was 14.9 ± 1.2‰ (± SD) for muscle and 14.6 ± 2.2‰ (± SD) for liver tissue. The lowest $\delta^{15}N$ values for stickleback muscle tissue were recorded in summer (June-July), with mean values between 14.1‰-14.3‰ (Fig. 2). During autumn and winter (October-February) intermediate values of 14.5%-15.2‰ were measured, and the highest values of between 15.3‰–15.7‰ occurred during spring in March and April (Fig. 2). Similar trends were observed for 815N in stickleback liver tissue, with lowest mean values occurring during the summer months (12.3%-12.4%), intermediate values from October to December (14.6%–15.7%) and highest values in March and April (16.1‰–16.6‰) (Fig. 2). δ^{15} N in the liver showed more monthly variation than muscle tissues in both littoral and pelagic habitats (F-tests on withingroup variations; $3.88_{185,180}$ and $3.25_{82,70}$ respectively, both $P \le 0.001$). Month had a significant influence, with the effect strength greatest in muscle tissue $\delta^{15}N$ (GLM, $r^2 = 0.23$, n = 252, P < 0.0001, Table 1). No other parameters had a significant effect (Table 1). The GLM for δ^{15} N values of liver tissue (r² = 0.60, n = 266) showed a similar result for month and year effects (P < 0.0001 and P < 0.01, respectively). The interaction between TL and month also significantly affected liver δ^{15} N, while no other parameters showed any significant effect (Table 1).

Mean δ^{13} C values averaged $-30.5 \pm 0.8\%$ (± SD) for stickleback muscle tissue and $-31.2 \pm 1.5\%$ (± SD) for liver tissue. Muscle δ^{13} C was lowest during July (-29.5‰) and fluctuated slightly between -30.1%—-31.0% (Fig. 2) during all other sampling months. Stickleback liver δ^{13} C values were highest during July (-28.5‰) and lowest during March (-32.2‰) and varied across other sampling months between -31.0%—-32.0% (Fig. 2). The model testing effects on δ^{13} C muscle values (GLM, r² = 0.27, n = 251, *P* < 0.0001) revealed that only month and sex had a significant influence (*P* < 0.0001). *Post hoc* comparison found that female fish had significantly lower muscle δ^{13} C values than males (Student-t, *P* < 0.05), but the effect strength of sex was 5× smaller than for month (Table 1). All other parameters had either no impact or exhibited only weak effects (Table 1). Month and sex had a significant influence (*P* < 0.0001) on liver δ^{13} C (GLM, r² = 0.46, n = 255, *P* < 0.0001), and as with muscle δ^{13} C level in muscle tissue, however, habitat had a significant effect (*P* < 0.05) on liver δ^{13} C, with significantly lower values in sticklebacks from the pelagic zone compared to littoral specimens (Student-t, to the pelagic zone compared to littoral specimens).



Figure 2. Fitted spline intervals of δ^{15} N and δ^{13} C in the muscle and liver of sticklebacks sampled in 2017–2018. Solid lines are the mean values, and shaded areas represent upper and lower 95% confidence intervals during the course of the year in Lake Constance.

Table 1. The significance and effect strength of study parameters on the $\delta^{15}N$ and $\delta^{13}C$ values of muscle and liver tissue of sticklebacks from Lake Constance.

δ^{15} N					
	muscle	liver			
parameter	effect strength (± standard error)	effect strength (± standard error)			
month	$0.96^{xxx} (\pm 0.005)$	$0.986^{xxx} (\pm 0.01)$			
habitat	$0.001 \ (\pm \ 0.001)$	0.007 (± 0.001)			
total length	0.247 (± 0.01)	0.059 (± 0.005)			
sex [m/f]	0.004 (± 0.001)	0.006 (± 0.001)			
infested [yes/no]	0.023 (± 0.001)	0.001 (± 0.002)			
parasite index	$0.001 (\pm 0.001)$	0.004 (± 0.001)			
TL*month	n.a.	n.a.			
	δ ¹³ C				
	muscle	liver			
parameter	effect strength (± standard error)	effect strength (± standard error)			
month	$0.513^{xxx} (\pm 0.01)$	$0.776^{xxx} (\pm 0.009)$			
habitat	$0.011 (\pm 0.001)$	$0.062^{x} (\pm 0.005)$			
total length	$0.187 (\pm 0.01)$	0.099 (± 0.006)			
sex [m/f]	$0.127^{xxx} (\pm 0.004)$	$0.146^{xxx} (\pm 0.007)$			
infested [yes/no]	0.0069 (± 0.003)	0.006 (± 0.005)			
parasite index	0.26 (± 0.007)	0.022 (± 0.003)			
TL*month	n.a.	n.a.			

Model terms: x = P < 0.05; xx = P < 0.01; xxx = P < 0.0001; effect strength is a dimensionless factor assessing the impact of a variable in the model formula.

P < 0.05). Overall, the effects of sex and habitat on the whole model were low (Table 1). There were no significant differences in the isotopic signature of sticklebacks caught in the pelagic and littoral habitat, except for the liver δ^{13} C values.

Stable isotope values of zooplankton, whitefish and sticklebacks (additional sampling)

The $\delta^{15}N$ value of zooplankton increased from October (10.6%) until December (13.4‰), showed the highest peak in February (14.4‰) and decreased until April to a value of 7.9‰ (Fig. 3). Eggs of pelagic whitefish showed a mean δ^{15} N value of $13.9 \pm 0.5\%$ (\pm SD) and did not differ significantly from those of benthic whitefish, which had a mean δ^{15} N value of 14.1 ± 1.1‰ (± SD) (t-test, P > 0.05). The combined mean δ^{15} N value for eggs of both species was 14.0 \pm 0.8‰ (\pm SD), significantly higher than that of zooplankton sampled in the same months (Fig. 3). The mean δ^{15} N value of sampled whitefish larvae, $15.3 \pm 0.2\%$ (\pm SD), was also higher than that for zooplankton sampled in the same months (Fig. 3). Sticklebacks sampled in 2021–2022 exhibited a mean δ^{15} N value of 15.4 \pm 0.5‰ (\pm SD) and a mean δ^{13} C value of $-31.0 \pm 0.6\%$ (\pm SD) and were close to results from winter 2017–2018. No differences were observed in mean $\delta^{15}N$ and $\delta^{13}C$ values between month and habitats (Tukey-Kramer-HSD, P > 0.0.5). The δ^{15} N values of sticklebacks showed no difference from those of whitefish larvae but were notably higher than those for zooplankton (Fig. 3). In March and April, when the zooplankton showed decreasing δ^{15} N values, the δ^{15} N values of sticklebacks continued on the same level as the month before (Fig. 3).

The δ^{13} C values of sampled zooplankton showed no clear temporal trend (-32.9% in October, -34.2% in December, -34.0% in February, -35.9% in March, and -34.8% in April). The mean δ^{13} C value of pelagic whitefish eggs was -33.7 ± 0.4% (± SD), significantly different to that of benthic whitefish eggs at -33.1 ± 0.6% (± SD) (t-test, *P* < 0.05). The mean δ^{13} C value of whitefish larvae was -33.2 ± 0.5% (± SD).

Stomach content analysis

Nearly all analysed sticklebacks had food in their digestive tracts. Only one individual sampled in the pelagic zone during spring had an empty stomach. The numerically dominant food source for sticklebacks during spring and winter, independent of sampled habitat, were copepods (Table 2). Interestingly, in 4 out of 10 sticklebacks (40%) sampled during spring in the littoral zone, fish eggs of unknown taxa were recorded, and in winter, the stomachs of two out of 20 (10%) pelagic sticklebacks contained fragments of fish eggs and partially digested fish larvae. Differences between the stomach contents of sticklebacks sampled in the pelagic or littoral zone became more obvious during other seasons. In summer, pelagic sticklebacks consumed mostly *Daphnia* (Table 2) while littoral sticklebacks fed mostly (73%) on benthic macroinvertebrates, mainly chironomids (here: other, Table 2). One stickleback caught in the littoral zone during summer had consumed 14 fish eggs of unknown taxa, amounting to 38% of all food items present in



Figure 3. Arithmetic mean δ^{15} N values with standard deviation of zooplankton, whitefish eggs and larvae and sticklebacks from the pelagic and littoral zone of Upper Lake Constance sampled in winter 2021–2022.

that individual's digestive tract. In autumn, copepods remained the most common prey category (81%) consumed by pelagic sticklebacks, whilst *Bosmina* (74%) were the most frequent prey category in littoral. Autumn was the only season when no fish (larvae or eggs) were found in either pelagic or littoral stickleback digestive tracts (Table 2).

Bayesian mixing model

The results of the SIMMR mixing model suggested a clear seasonal distinction in the contribution of food sources to sticklebacks (Fig. 4). During winter, whitefish larvae and whitefish eggs contributed a mean proportion of 30–40% (the highest contribution) to the diet, while zooplankton and chironomids are of lesser importance (mean values between 10–15%), independent of sampling habitat (littoral or pelagic zone) (Fig. 4). During summer, the importance of chironomids and zooplankton increased to proportions of 25–30% (chironomids) and 35–40% (zooplankton). During summer, neither whitefish eggs nor whitefish larvae are available. However, other proteinrich sources, *i.e.* fish larvae from later spawning species, increased in importance during that time to provide a mean value of 30% (pelagic zone) and 40% (littoral zone), compared to winter, when fish (non-whitefish) contribute a proportion of around 15% to the diet of sticklebacks (Fig. 4). When we excluded the category "Fish" from the model, the importance of chironomids and plankton increased during summer to proportions of 30–35% for chironomids and 65–70% for zooplankton (Suppl. mate-

Table 2. Diet composition of sticklebacks (prey types expressed as a percentage of the total number of
prey items eaten: during spring, summer, autumn and winter, and as the mean number of consumed
individuals per stickleback \pm SD) sampled in pelagic and littoral zone of Upper Lake Constance. h/d
cladocera = other herbivorous/detritivorous cladocera.

habitat	spring	summer	autumn	winter
pelagic	85.95%	12.12%	81.44%	56.06%
	371 ± 254	124 ± 215	596 ± 304	172 ± 76
littoral	58.18%	12.49%	15.50%	68.98%
	124 ± 215	7 ± 7	28 ± 36	122 ± 158
pelagic	2.41%	5.63%	6.82%	0.13%
	10 ± 7	7 ± 8	50 ± 36	1 ± 1
littoral	0.67%	6.54%	73.83%	1.13%
	1 ± 2	2 ± 3	111 ± 74	1 ± 1
pelagic	10.66%	78.83%	11.47%	43.69%
	46 ± 29	96 ± 108	84 ± 71	134 ± 61
littoral	0.96%	4.16%	2.41%	13.55%
	1 ± 3	2 ± 1	2 ± 2	17 ± 23
pelagic	0.05%	3.00%	0.27%	0.06%
	1 ± 1	4 ± 5	4 ± 4	1 ± 0.6
littoral	0.00%	0.36%	0.04%	0.11%
	0	1 ± 0	1 ± 0	1 ± 0
pelagic	0.00%	0.00%	0.00%	0.03%
	0	0	0	1 ± 0
littoral	2.34%	3.78%	0.00%	0.00%
	1 ± 1	1 ± 4	0	0
pelagic	0.93%	0.41%	0.00%	0.02%
	5 ± 5	1 ± 1	0	1 ± 0
littoral	37.84%	72.67%	8.22%	16.22%
	13 ± 8	35 ± 29	4 ± 11	7 ± 5
	habitat pelagic littoral pelagic littoral pelagic littoral pelagic littoral pelagic littoral pelagic littoral	habitat spring pelagic 85.95% 371 ± 254 littoral 58.18% 124 ± 215 pelagic 2.41% 10 ± 7 littoral 0.67% 1 ± 2 pelagic 10.66% 46 ± 29 littoral 0.96% 1 ± 3 pelagic 0.05% 1 ± 1 littoral 0.00% 0 0 pelagic 0.00% 0 0 pelagic 0.00% 0 0 pelagic 0.93% 5 ± 5 5 ± 5 littoral 37.84% 13 ± 8 2 ± 8	habitat spring summer pelagic 85.95% 12.12% 371 ± 254 124 ± 215 littoral 58.18% 12.49% 124 ± 215 7 ± 7 pelagic 2.41% 5.63% 10 ± 7 7 ± 8 littoral 0.67% 6.54% 1 ± 2 2 ± 3 pelagic 10.66% 78.83% 46 ± 29 96 ± 108 littoral 0.96% 4.16% 1 ± 3 2 ± 1 pelagic 0.05% 3.00% 1 ± 1 4 ± 5 littoral 0.00% 0.36% $0 = 0$ 1 ± 1 4 ± 5 littoral 0.34% 3.78% $0 = 0$ 0 0 pelagic 0.93% 0.41% $pelagic$ 0.93% 0.41% 1 ± 1 1 ± 4 $pelagic$ 5 ± 5 1 ± 1 1 ± 1 1 ± 1	habitatspringsummerautumnpelagic 85.95% 12.12% 81.44% 371 ± 254 124 ± 215 596 ± 304 littoral 58.18% 12.49% 15.50% 124 ± 215 7 ± 7 28 ± 36 pelagic 2.41% 5.63% 6.82% 10 ± 7 7 ± 8 50 ± 36 littoral 0.67% 6.54% 73.83% 1 ± 2 2 ± 3 111 ± 74 pelagic 10.66% 78.83% 11.47% 46 ± 29 96 ± 108 84 ± 71 littoral 0.96% 4.16% 2.41% 1 ± 3 2 ± 1 2 ± 2 pelagic 0.05% 3.00% 0.27% 1 ± 1 4 ± 5 4 ± 4 littoral 0.00% 0.36% 0.04% 0 1 ± 0 1 ± 0 1 ± 0 pelagic 0.00% 0.00% 0.00% 0 0 0 0 pelagic 0.00% 0.00% 0 0 0 pelagic 0.93% 0.41% 0 1 ± 1 1 ± 4 0 1 ± 1 0 1 ± 1 1 ± 4 0 pelagic 0.93% 0.41% 0 0 0 1 ± 1 1 ± 1 0 <t< td=""></t<>

rial 1: fig. S2), however, during winter, the importance of whitefish eggs and larvae was (with a mean contribution of 30-45% to the diet) similar to the outcome of the model which included the category "Fish" (Suppl. material 1: fig. S2). The detailed summary of both Bayesian mixing models (SIMMR) outputs and matrix plots of source contribution proportions are given in the supplements (Suppl. material 1: tables S1–S4).

Stable isotope values of other fish species from Lake Constance

For the other fishes species examined from Lake Constance, mean $\delta^{15}N$ values for muscle tissue varied by up to 4.0‰, with a minimum of $9.6 \pm 1.1\%$ for herbivorous rudd and a maximum of $13.6 \pm 0.1\%$ for piscivorous pike with whitefish, bleak, roach, burbot, and tench exhibiting intermediate values (Fig. 5). At the time of sampling (summer), mean stickleback muscle $\delta^{15}N$ (14.3 \pm 1.2‰) did not differ from that of exclusively piscivorous pike (Tukey-Kramer-HSD, P > 0.05) and was significantly higher than that of all other fish species (Tukey-Kramer-HSD, P < 0.0.5). The δ^{15} N values of guagga mussels and faucet snails were $5.6 \pm 0.1\%$ and $6.9 \pm 0.2\%$ respectively (Fig. 5).



Figure 4. Posterior distribution of dietary proportion estimates of different food sources from sticklebacks from the pelagic and littoral zone of ULC during summer and winter, according to Bayesian modelling, expressed as Box-and-Whisker plots with median values and interquartile range (IQR), and minimum and maximum if it doesn't extend the IQR value beyond 1.5. Data outside this range are plotted individually.

The mean muscle δ^{13} C of all analysed fish species, except sticklebacks, ranged from -29.6 ± 1.4‰ for whitefish to -21.4 ± 1.9‰ for rudd, while mean values for bleak, roach, tench, pike and burbot ranged between -28.4‰ and -25.1‰ (Fig. 5). At the time of sampling, in summer, the mean muscle δ^{13} C of sticklebacks (-30.5 ± 1.0‰) was significantly lower than that of all other fish species (Tukey-Kramer-HSD, P < 0.0.5). The δ^{13} C values of quagga mussels and faucet snails were -30.1 ± 0.3‰ and -24.3 ± 0.9‰ respectively (Fig. 5).

Fig. 5 shows the core isotopic niche of each feeding guild. SEAc values range from 2.43 (zooplanktivorous) to 6.56 (benthivorous). The niche overlap between omnivorous sticklebacks and other feeding guilds was limited to a 9% overlap with zooplanktivorous whitefish.

Trophic position of sticklebacks

The statistical model testing effects on the stickleback trophic position (GLM, $r^2 = 0.29$, n = 249, P < 0.0001) identified a significant influence of month (P < 0.0001), which comprised > 64% of total effect strength revealed the model (Table 3). When using a Δ_n value of 3.4, the mean trophic position of sticklebacks in Lake Constance was $4.7 \pm 0.6 (\pm \text{SD})$. The reduced Δ_n value of 2.0 yielded stickleback trophic positions of 6.7 \pm 1.0 (\pm SD). This is unrealistic given the food web and feeding guilds of Lake Constance. Consequently, further analyses of trophic position are based solely



Figure 5. δ^{15} N versus δ^{13} C bi-plot showing the mean isotope values of aquatic consumers in Lake Constance during summer (August and September). Horizontal and vertical bars represent \pm SD of total pooled data. The standard ellipse areas (SEAc) represent the core isotopic niche for each trophic guild (comprising ~ 40% of the data; (Jackson et al. 2011)).

on results from the $\Delta_n = 3.4$ iterations of the model. The lowest trophic positions for sticklebacks were calculated for the summer months of June and July (4.4–4.6) and the highest for the spring months of March and April (4.9–5.0). No other tested parameters had a significant effect on trophic position (Table 3).

The trophic position for piscivorous pike in ULC was 4.2 \pm 0.2 (\pm SD) and for partly piscivorous burbot 4.2 \pm 0.4 (\pm SD).

Table 3. The significance and effect strength of different parameters on the trophic position of sticklebacks in Lake Constance.

parameter	effect strength		
month	$0.969^{xxx} (\pm 0.005)$		
habitat	$0.001 \ (\pm \ 0.001)$		
total length	0.235 (± 0.01)		
sex [m/f]	$0.001 \ (\pm \ 0.001)$		
infested [yes/no]	$0.012 (\pm 0.001)$		
parasite index	$0.011 (\pm 0.0011)$		
TL*month	n.a.		

Model terms: x = P < 0.05; xx = P < 0.01; xxx = P < 0.0001; effect strength is a dimensionless factor assessing impact of variable in model formula.



Figure 6. Fitted spline intervals of the trophic position of sticklebacks in Upper Lake Constance. Solid lines are the mean values, and shaded areas represent upper and lower 95% confidence intervals during the course of the year.

A seasonal trend in stickleback trophic position was apparent, with lowest values during the stickleback spawning season (summer months), and increasing in the spawning season of whitefish during autumn and winter (Fig. 6).

Trophic position of sticklebacks in other lakes and in comparison to Lake Constance

Sticklebacks from Lake Constance have a significantly higher trophic position than investigated populations in North America and Norway (z-test, P < 0.001) (Fig. 7). The proportion of littoral carbon in the diet of sticklebacks (α) from Lake Constance (mean = 0.06, standard error = 0.01) is comparable with that observed in populations with a "limnetic-like" phenotype from Norway and North America ($\alpha \le 0.4$; Fig. 7) but far from that documented for benthic populations ($\alpha \ge 0.6$; Fig. 7).

Discussion

A key insight from the present study is the seasonal trend in $\delta^{15}N$ from stickleback muscle and liver, with the highest values occurring during winter and spring. These values assist us to answer research question 1 (Do the $\delta^{15}N$ values and trophic position of sticklebacks reflect the seasonal feeding of whitefish larvae and eggs or that of



Figure 7. Comparison of trophic position and littoral carbon in the diet of three-spined sticklebacks. Values for sticklebacks from Lake Constance in black (mean value of all analysed sticklebacks, independent of habitat), from North America in blue and Norway in red; error bars indicating standard deviation. The eight North American stickleback populations (Matthews et al. 2010) (PRL = Priest Lake, KL = Kennedy Lake, PAL = Paxton Lake, CL = Cranby Lake, DL = Dugout Lake) and the two from Norway (Østbye et al. 2016) (EP = Einletvatn pond, FL = Farstadtvatn lake) are subdivided into limnetic (n = 4), benthic (n = 4) and intermediate (n = 1) ecophenotypes.

other food sources, such as zooplankton?). It could be hypothesised that the seasonal trend is linked to elevated δ^{15} N values of the main food resource during winter (here copepods and cladocera), as observed in Lake Geneva where whitefish consume mainly zooplankton during the winter months (Perga and Gerdeaux 2005, 2006). There, the seasonal nature of zooplankton δ^{15} N was echoed in the tissues of whitefish after a lag of one month in the case of liver tissue, and four to five months later in the case of muscle tissue. Sticklebacks from Lake Constance also consume large quantities of zooplankton (Ogorelec et al. 2022). However, it appears that stickleback δ^{15} N values are only partly dictated by the isotopic signature of zooplankton. On the one hand, we can see a winter increase and decrease in the δ^{15} N values of stickleback liver (as in Lake Geneva) and observe a switch from the summer diet of *Daphnia* and macrozoobenthos to copepods during winter. Much the same diet shift and increases in δ^{15} N were observed in a study from North America (McIntyre et al. 2006). On the other hand, δ^{15} N values in stickleback tissue from ULC increase up to 16.6‰ and are distinctly higher than those observed in local zooplankton (7.9%-14.4%). Moreover, values in stickleback muscle (which typically respond more slowly to dietary change) decreased only slightly after winter, and never fell below 14.1‰ throughout the year. In contrast, the δ^{15} N value of zooplankton decreased to below 8‰ in April. Therefore, seasonal changes in zooplankton feeding from *Daphnia* to copepods alone cannot be responsible for the distinctly elevated δ^{15} N values observed in ULC sticklebacks during winter or the moderately high values (well above those recorded in other fish species) observed during the rest of the year. Were that the case, we would expect to observe much lower $\delta^{15}N$ values yearround and a much stronger decrease after winter. Additionally, during summer, the mean δ^{15} N value of stickleback muscle (14.3 \pm 1.2‰) was significantly higher than that of adult whitefish $(13.2 \pm 0.5\%)$, indicating differences in feeding ecology. Our stomach content analyses partially support the stable isotope results, as in winter the stomachs of 20% of pelagic sticklebacks contained fragments of fish eggs and partially digested fish larvae, while in spring 40% of littoral sticklebacks sampled contained fish eggs of unknown taxa. Judging by the season and pigmentation, the eggs and the larvae most likely belong to whitefish. Furthermore, stable isotope analysis of whitefish eggs and larvae revealed much higher $\delta^{15}N$ levels than were identified in zooplankton. Thus, it is highly likely that the δ^{15} N increase in the liver of sticklebacks during winter and spring and the high δ^{15} N values in the muscle during spring are at least partly due to piscivory. Moreover, the outcome of our stable isotope mixing models estimates that whitefish larvae and whitefish eggs were the dominant prey items of sticklebacks during winter. This outcome suggests that we severely underestimate the predation rate during winter using the stomach content analysis alone. We guess that two factors are responsible for this outcome: first, the stormy winds during and after the spawning seasons, which hampered a normal sampling and second, the extremely patchy distribution of whitefish larvae (Ransom et al. 2022) and therefore clutched appearance of larvae in the diet of sticklebacks, which is further supported by mass feeding (Roch et al. 2018). It could be hypothesised that during food scarcity in winter, when the density of zooplankton and benthic macroinvertebrates is much lower compared to summer (Ogorelec et al. 2022), whitefish eggs and larvae are an exceptionally valuable additional diet component for sticklebacks due to their easy availability and undisputed caloric value (Ros et al. 2019; Baer et al. 2021). Furthermore, the Bayesian model calculated for summer a proportion of 30% (pelagic zone) and 40% (littoral zone) of fish in the diet of sticklebacks. As for winter, this outcome starkly contrasts with our stomach content analysis. We found only one female stickleback, caught in August, which had eaten 14 fish eggs. However, even if cannibalistic behaviour is well known for sticklebacks (FitzGerald and van Havre 1987; Mehlis et al. 2010), those eggs were highly likely not from sticklebacks, because sticklebacks in ULC spawn much earlier, between April and June (Gugele et al. 2020). Different cyprinids, like bleak Alburnus alburnus and the white bream Blicca bjoerkna, are multiple spawners and some spawn until the end of July (Rinchard and Kestement 2003). Thus, it seems likely that sticklebacks from ULC consume, if the opportunity arises, eggs from other fish species, *i.e.* cyprinids, as has been observed in other water systems before (Dukowska and Grzybkowska 2014). Furthermore, nearly all fish from ULC spawn in the littoral zone, not in pelagic waters. Therefore, the difference in fish consumption from sticklebacks caught during summer in both habitats most probably reflects the availability of fish eggs and larvae in both habitats. All in all, the results from the stable isotope mixing models pointed to a significant piscivorous behaviour of sticklebacks in ULC. It is well known from other parts of the world that sticklebacks can consume high amounts of fish seasonally: in the Baltic Sea sticklebacks were observed to migrate purposely to the spring spawning grounds of perch and pike, where they fed on eggs and larvae of those species. This leads to significant stock reductions of the latter two species (Ljunggren et al. 2010;

Bergström et al. 2015; Byström et al. 2015). In ULC, similar patterns were observed

(sticklebacks migrated to the spawning grounds of whitefish, Gugele et al. 2020). The answers to research questions 2 (Where do invasive sticklebacks sit relative to other fish species in the trophic structure of Lake Constance?) and 3 (How does the trophic position of sticklebacks in Lake Constance compare to that of conspecifics in similar ecosystems?) are somewhat contradictory. Generally, Lake Constance sticklebacks occupied an extraordinarily high trophic position (mean = 4.7), even using a conservative estimate of trophic enrichment ($\Delta_{1} = 3.4$). Other studies, using the same calculation and the same values for trophic enrichment and trophic position of the consumer ($\lambda_{Base} = 2$), yielded markedly lower scores: The mean trophic positions calculated for sticklebacks in lakes of North America and Norway ranged from 2.9 to 3.7 (Matthews et al. 2010; Østbye et al. 2016), and in Japan, the species occupies a mean trophic position of 2.8 (Ravinet et al. 2014), however, in those lakes no piscivorous behaviour of sticklebacks was observed. In large oligotrophic lakes, trophic positions higher than 4 are normally occupied by at least partly piscivorous fish like arctic charr (Salvelinus alpinus L., 1758) or perch (Perca fluviatilis L., 1758) (Eloranta et al. 2015). In ULC, pike and burbot showed trophic positions of around 4.2, and their mean $\delta^{15}N$ values are highly comparable to sticklebacks. However, this does not mean that they were all at the same trophic level because it is proven that the intra- and interindividual variation in the isotopic composition depends not only on the diet, but also on the amount of isotopic variations among the food sources (Matthews and Mazumder 2005). We found large seasonal variations in the isotopic signatures of zooplankton in ULC as other authors did (Perga and Gerdeaux 2006; Janjua and Gerdeaux 2011). In those cases, modelling the trophic position of fish species is hardly reliable because many sources of $\delta^{15}N$ variation are, therefore, unrelated to trophic variation (Matthews and Mazumder 2005; Janjua and Gerdeaux 2011). Another explanation for the high trophic position of sticklebacks in ULC sticklebacks could be the δ^{15} N or δ^{13} C values (here: baseline values) of other consumers represented here by faucet snail and quagga mussel. It is known that interannual variation in the δ^{15} N or δ^{13} C values of snails and mussels can occur (Westrelin et al. 2023). We sampled snails and mussels two years after we sampled the sticklebacks which may have biased our outcomes. Furthermore, it is known that the $\delta^{15}N$ or $\delta^{13}C$ values for dreissenids in Lake Constance (zebra mussel Dreissena polymorpha Pallas, 1771), are depth, water temperature and season dependent (Yohannes et al. 2014). However, even if we use this seasonal variation to consider potential innerannual variation and to adjust the δ^{15} N or δ^{13} C values for the season (late summer) to a range of -30.5‰ to -34.5‰ for δ^{13} C and 6‰ to 9‰ for δ^{15} N (cf. Yohannes et al. 2014) for the baseline values,

stickleback trophic position always exceeds 4. In other lakes, for example, the western basin of Lake Erie in the US, δ^{13} C values of around -22‰ were reported for guagga mussels (Guzzo et al. 2011). Those values would lead, even in Lake Constance, to a different α -value, and a trophic position of around 3.5 and are, thus, very comparable to other lakes in North America (Matthews et al. 2010). However, the western basin of Lake Erie is much warmer than Lake Constance and mesotrophic (Guzzo et al. 2011), and it is generally known that $\delta^{15}N$ values in warmer ecosystems are generally lower while δ^{13} C values tend to be elevated in cooler systems (Yohannes et al. 2014). These factors must be considered when looking to trophic position results for sticklebacks from other parts of the world, even if they are acquired using the same equation (Post 2002) and the same method. Ecosystems operating under different temperature regimes may not be strictly comparable. However, comparisons between this study and those from Canadian and Norwegian lakes with similar cooler temperature regimes are valid, and by these benchmarks, the trophic position of stickleback in Lake Constance is remarkably high. Further investigations are needed to disentangle the effects of temperature on stable isotope turnover rates and diet-tissue discrimination patterns.

The diet and stable isotope mixing model evidence of regular or at least occasional piscivory by Lake Constance sticklebacks are corroborated by comparison of the mean δ¹⁵N muscle values of sticklebacks with those of fish species from a range of foraging guilds. Sticklebacks possessed significantly higher muscle δ^{15} N values (up to 5‰) than the zooplanktivorous and/or benthivorous and herbivorous fish in our analysis, including whitefish, bleak, roach, tench, and rudd. Other studies have shown $\delta^{15}N$ differences between years for various fish species, but the values of the fish species from one foraging guild tend to be more or less stable from year to year (Janjua and Gerdeaux 2011). Furthermore, mean stickleback 815N muscle values in Lake Constance are much the same as those of pike here and in other lakes (Juanes et al. 2002; Craig 2008). This indicates that a considerable portion of stickleback diet is derived from protein-rich prey (Gu et al. 1996; Frisch et al. 2014), such as fish eggs or larvae. Recent studies of sticklebacks in Lake Constance in which stomach content analysis identified zooplankton as the only prey (Bretzel et al. 2021; Ogorelec et al. 2022), should be scrutinised in the light of these findings, as indigestible arthropod body parts will remain longer in alimentary tract than protein-rich prey items (Preston et al. 2017). And the extremely patchy distribution of whitefish larvae (Ransom et al. 2022) makes regular feeding highly doubtful. Not only the present study but also other studies dealing with invasive fish species have shown that stable isotope analysis, together with the modelling of contribution rate of potential food sources to consumers, can provide a more effective assessment of an invader's feeding behaviour and potential environmental impacts than classical stomach content analysis alone (Brush et al. 2012).

The answer to research question 4 (Are there any differences in isotopic signature between sticklebacks caught in littoral and pelagic habitats?), if only the δ^{13} C values recorded in stickleback muscle tissue are considered, is relatively clear: the answer is no. The low values (-30.5 ± 0.8‰) point to pelagic feeding of the species (France 1995). The apparently low proportion (<10%) of littoral carbon (α) assimilated from the diet

of ULC sticklebacks strongly suggests that the population comprises predominantly pelagic feeders. In benthic-feeding individuals of this species, the proportion of littoral carbon is normally around 60% or higher (Matthews et al. 2010; Østbye et al. 2016). However, it is somewhat surprising that littoral habitat has so little influence on stickleback muscle 813C values, as stomach content analysis suggests that during summer and autumn, littoral and pelagic sticklebacks consume distinctly different diets: significantly, dietary overlap was only observed in winter and spring (Table 3). Muscle tissue is typically favoured in stable isotope analysis to characterise longer-term feeding patterns and to differentiate consumers into ecotypes or guilds (Matthews et al. 2010; Østbye et al. 2016), while the rapid turnover of liver tissue means it incorporates and processes diet components faster than muscle tissue and is thus more sensitive to shortterm feeding changes (Perga and Gerdeaux 2006). These trends are borne out in the current study, where a seasonal habitat shift appears to significantly affect $\delta^{13}C$ liver levels but not muscle tissue δ^{13} C levels. It may be that the duration of littoral feeding is too short to register in muscle or that a potentially reduced feeding rate during the littoral phase (during/after spawning in summer and autumn) is compensated by an increased feeding rate during the pelagic phase (winter and spring), or that an interplay of these effects occurs. Thus, further studies of stable isotope signatures of the liver will be needed to ascertain which of these three possibilities is the case and also to further test the recent theory that a benthic-pelagic species pair (one littoral, one pelagic, cf. Dahms et al. 2022) exist in Lake Constance. The latter question may require the deployment of specific markers, such as fatty acids (Hou et al. 2020; Rubenson et al. 2020) or sophisticated genetic analysis (Margues et al. 2016). Further laboratory studies are also required to attain more detailed and species-specific information regarding the effects of different diets (zooplankton, fish eggs, fish larvae, etc.), the impact of starvation, spawning, and parental care on the isotopic expression in stickleback muscle and liver tissue. The hypothesis that stocking with recently hatched whitefish larvae (as has been the practice in ULC for over a century) facilitates stickleback population growth and spread (Roch et al. 2018) also requires further investigation in order to ascertain whether stocking is a factor prompting the stickleback invasion of the pelagic zone in ULC.

Results of this study pertinent to the original research questions include confirmation from stomach content analysis that sticklebacks feed on whitefish larvae and eggs as well as fish of unknown taxa: corroborated by δ^{15} N values, the outcome of stable isotopes mixing models, and trophic profiling. Stable isotope analysis revealed significantly elevated δ^{15} N values comparable to those of pike and consistent with piscivory, and δ^{13} C profiles identified stickleback as mostly pelagic feeders. Furthermore, it appears that while the trophic position of sticklebacks is independent of their littoral and pelagic foraging habitats, differences in the isotopic signature of littoral and pelagic captures were visible in the liver, offering support for the idea that stickleback has a key role coupling the littoral and pelagic food webs of Lake Constance. However, actual data (not shown) of stomach contents of piscivorous fish, such as pike, catfish, char or trout (*Salmo trutta*), revealed that sticklebacks were only eaten occasionally by other fish and stickleback predation seems instead to be mostly from fish-eating birds, which would render this avenue a dead end for the aquatic ecosystem. However, a similar assessment was hypothesized for the dreissenid mussels in the Great Lakes, where the lakewide degree of mussel predation by fish was believed to be limited, but when round goby (*Neogobius melanostomus*) and lake whitefish (*Coregonus clupeaformis*) began to feed intensively on quagga mussels they contributed significantly to Great Lakes' food webs (Madenjian et al. 2010). Whether the predatory fish of Lake Constance will adapt to the stickleback resource, especially in the pelagic zone, is an open question for the future.

Conclusion

The study results support the hypothesis that Lake Constance sticklebacks feed occasionally but rather intensively on the eggs and larvae of whitefish and some other fish species. This finding is in line with findings from the Baltic Sea, which highlight the negative impacts of stickleback predation on other fish species (Ljunggren et al. 2010; Bergström et al. 2015; Byström et al. 2015). The data in this study sheds important light on the puzzle of the Lake Constance stickleback invasion, in particular the question of how they have become such an important component of the food web of this large ecosystem. Our results suggest that sticklebacks in ULC are predators of whitefish larvae and eggs with significant negative consequences for their recruitment, and that this behaviour should be considered in the future management of the lake.

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Supplementary material I

Supplementary information

Authors: Sarah Maria Gugele, Jan Baer, Christina Spießl, Elizabeth Yohannes, Steve Blumenshine, Barnaby J. Roberts, Mario R. Mota-Ferreira, Alexander Brinker Data type: docx

- Explanation note: table S1. Quantiles of the posterior distribution of the Bayesian mixing model parameter estimation. table S2. Correlation of the posterior distribution of the Bayesian mixing model parameter estimation. figure S1. Isospace plot representing the isotopic signatures of the stickleback liver samples caught in summer (June and July) and in winter (December to March). table S3. Quantiles of the posterior distribution of the Bayesian mixing model parameter estimation for models without the fish sources. table S4. Correlation of the posterior distribution of the Bayesian mixing model parameter estimation for models without the fish sources. table S4. Correlation for models without the fish sources. figure S2. Posterior distribution of dietary proportion estimates of different food sources from sticklebacks from the pelagic and littoral zone of ULC during summer and winter, according to Bayesian modelling without fish sources, expressed as Boxand-Whisker plots with median values and interquartile range (IQR).
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RESEARCH ARTICLE



Capsaicin-treated bait is ineffective in deterring non-target mammals from trap disturbance during invasive lizard control

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Abstract

Excluding non-target species from invasive species control efforts can be challenging due to non-target attraction to trap structure, baits, and lures. Various methods have been used to deter non-target species from entering or disturbing traps including altered features (e.g., mesh size, trip mechanism, or entrances), staking traps, and chemical deterrents. Invasive populations of Argentine Black and White Tegu lizards (Salvator merianae) occur in several locations across Florida and Georgia, and there are ongoing trapping efforts to control them. At sites in Georgia, non-target mammals disturb most of the lizard traps (>80%), consume egg bait/lures, and thus reduce trap efficacy. In contrast, our Florida site has fewer problems with non-target mammals. Our goal was to quantify the efficacy of capsaicin-coated eggs, a known distasteful irritant to mammals, as a non-target bait deterrent in live traps set for tegus in both Georgia and Florida. We conducted feeding assays on three tegus and found that individuals readily consumed food coated in capsaicin. We then conducted a three-part, live trapping experiment to test 1) if trap disturbance by mammals habituated to eggs without capsaicin decreased when capsaicin-coated eggs were deployed in Georgia, 2) if mammals not habituated to eggs as bait (treated or untreated) disturbed live traps at the same rate as those habituated to eggs in Georgia, and 3) if tegu capture rates were different when capsaicin treated eggs were deployed in Florida. In Georgia, we found that trap disturbance by non-target mammals did not decrease when capsaicin was applied to eggs in an area previously habituated to trapping with this bait nor when applied in a novel area. In Florida, we found no significant difference in tegu captures using capsaicin-treated vs. untreated bait. Tegus were tolerant of capsaicin, but capsaicin treated eggs did not reduce non-target mammal disturbance to traps. Therefore, removal of invasive populations could be problematic if methods to reduce trap disturbance by non-targets are not identified and deployed.

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Keywords

deterrents, invasive species, live trap, non-target species, Salvator merianae, Tegu lizards

Introduction

Early detection and rapid response (EDRR; Westbrooks 2004; Reaser et al. 2020) to invasive species depends on effective sampling methodologies to detect, identify and/ or capture target species (Morisette et al. 2020). Yet, any effects on non-target species must also be minimized. Traps are often used to remove invasives, however the specificity, efficiency, and cost of trapping efforts presents hurdles to invasive species management (e.g., Rodda et al. 1999). For EDRR efforts in particular, the consequences of not overcoming these trapping hurdles are potentially significant and can lead to a failure to eradicate the invasive population.

Excluding non-target species is challenging due to the variety of potential interactions species may have with traps. Non-target species may be attracted to traps as refugia or because a trap is placed in an area it forages (e.g., Peitz et al 2001). Likewise, non-target species may simply investigate, then trigger, the trap such that the target species will be excluded until the trap is reset. Whether a non-target species is captured (bycatch) or simply disturbs a trap, the result is a loss of trapping opportunity for target species. Lost trap opportunity thereby increases the time and cost of the trapping effort of the target species, making it harder and/or longer to achieve EDRR goals (e.g., delineate invasive species' population, eradication).

A variety of approaches have been used to deter nuisance species from an area or resource (Young et al. 2019; Werrell et al. 2021) and non-target species from traps (Peitz et al. 2001). Bycatch-reduction methods are common and include altered mesh size on traps or seines (Bohnsack, et al. 1989), or turtle excluder devices on trawling nets (Jenkins 2012). A variety of traps might be physically modified to alter the entrance so that greater specificity for the size and shape of the target species is achieved (Roden-Reynolds et al. 2018), or adjustment of the trip mechanism for a targeted species weight (or behavior) (Haro et al. 2020). Chemical attractants (Kimball et al. 2000; Landolt 2000) and deterrents (Kimball et al. 2009; Baylis et al. 2012; Burke et al. 2015; Lei and Booth 2017) have also been applied to nets, traps, or bait to increase trap specificity or to reduce the disturbance of traps by non-target species.

Increasingly, non-native reptile species are being introduced via human movement of goods or the pet trade (Lockwood et al. 2019; Orzechowski et al. 2019; Mazzamuto et al. 2021). Invasive Argentine Black and White Tegu lizards (hereafter, tegus; *Salvator merianae*) have been reported in 35 counties in Florida and at least four breeding populations have been established (Harvey et al. 2021). Tegus have broad habitat (Jarnevich et al. 2018), thermal (Currylow et al. 2021; Goetz et al. 2021), and dietary (Offner et al. 2021) requirements. Also, tegus have been documented eating threatened or endangered species such as hatchling gopher tortoises in Florida (*Gopherus polyphemus*; Offner et al. 2021). In Georgia, numerous tegus have been trapped in two counties (Toombs, Tattnall; Haro et al. 2020), and in July 2019, a live-trapping program for tegus was initiated at the primary site of captures and reports in these two counties. Following protocols developed by the U.S. Geological Survey and the National Park Service for tegu trapping in Florida (described in Udell et al. 2022), live traps were baited with chicken eggs to capture tegus in Georgia. Almost immediately, tegu traps were disturbed by non-target species such that concerns arose that trapping efficiency was seriously reduced. Hence, solutions were sought to identify species that disturbed the traps, and to reduce trap disturbance.

Here, we document trap disturbance by non-target species and quantify the efficacy of using capsaicin-coated eggs as bait in live traps set for Argentine Black and White Tegus. Capsaicin is extracted from *Capsicum* plants and is both distasteful and an irritant to mammals (Osborn and Rasmussen 1995; Tewksbury and Nabhan 2001). Birds, however, tolerate capsaicin because they lack a functional receptor for it (Jordt and Julius 2002; Baylis et al. 2012). Given that birds are diapsid reptiles descendant from lizard-like therapods (i.e., extant birds and reptiles share a more-recent common ancestor than either group does with extant mammals), we suspect lizards may tolerate capsaicin. We conducted feeding assays on three tegus to quantify if tegus negatively responded to food coated in capsaicin. We then conducted a three-part, live trapping experiment to test 1) if trap disturbance by mammals habituated to eggs (bait) without capsaicin decreased when capsaicin-coated eggs were introduced, 2) if mammals not habituated to eggs as bait (treated or untreated) disturbed live traps at the same rate as those habituated to eggs, and 3) if tegu capture rates were different when capsaicin treated eggs were used.

Methods

Feeding trials

To evaluate if capsaicin-treated food items would deter tegus from feeding, feeding trials were recorded by presenting a tegu with odiferous, desirable food. Three subjects were fed between 28 May 2020 and 19 September 2020. Each subject was tested either 4 or 5 times, with two or more days separating feeding trials. Two tegus were long-term captive pets, and a third was wild caught but also a long-term captive animal. Each tegu was offered capsaicin-treated food item, then control food item, then capsaicin-treated food item again until the food ran out, or the lizards refused to eat any more food for more than two minutes. The order of treated / untreated food presented to each tegu was randomized at the start of each feeding trial.

Two lizards were fed raw chicken breast cut into approximately 6.45 cm² cubes. One lizard was also fed Vienna sausages (one can) because this was a highly desirable food item provided to it by its owner. Capsaicin-treated food was coated in

approximately 2 mL of a capsaicin solution. To make the solution, we dissolved 0.12 g of a commercial food additive (Mad Dog 357 Yellow Cake Capsicum Powder) per mL of distilled water. Mad Dog 357° Yellow Cake Capsicum Powder (hereafter "capsaicin powder") advertises as 10% capsaicinoids by weight and 1,600,000 Scoville as determined through high-performance liquid chromatography. Control food items were moistened with tap H_2O . To facilitate data collection, each feeding trial was recorded on a smart phone with the lizard's head in frame. Tongs were used to offer food items to the lizard.

Trapping

In May 2019, an incipient population of tegus was identified in south eastern Georgia, and live trapping began for their removal (Haro et al. 2020). The following year, live trap arrays were opened in Georgia on 17 March 2020 (site A, experiment 1), 18 March 2020 (site J, experiment 1), and on 26 April 2021 (site D, experiment 2; Fig. 1). Trap disturbance by non-target species rapidly increased thus we sought to evaluate non-target species response to capsaicin at sites with prior tegu trapping effort in 2019 (sites A and J) and sites without prior tegu trapping efforts (site D). Live trap arrays were opened in Florida (Miami-Dade) on 8 July 2021 (site F, experiment 3) to assess capsaicin treatment on tegu captures. Sites in Georgia used modified Havahart (Havahart, Inc., Lititz, PA, hereafter "live traps") traps of small ($L \times W \times H$: 44.5 cm \times 14.6 cm \times 18.3 cm) and medium (L \times W \times H: 63.5 cm \times 17.8 cm \times 18.4 cm) sizes. Traps were modified by wrapping hardware cloth (0.64 cm mesh; dimensions $L \times W$: 43.2 cm × 33 cm [small] and 63.5 cm × 48.3 cm [medium]) to prevent escape of juvenile tegus. When deployed, one door was kept closed to leave a single entrance. Traps were staked down by two 26-inch lengths of rebar positioned so they did not disturb the trap's operation or tegu entry and were concealed as much as possible with leaf litter or sand. Traps at site A and D were deployed in a grid with 50 m spacing between traps. At site J, traps were placed 50 m apart in a linear array adjacent to an unpaved roadway. At site D, traps were spaced 100 m apart in four lines (5 traps per line), and lines were separated by 50 m. At site F, small live traps (similarly modified with hardware cloth) were deployed in a linear array along the verge and tree line of an unpaved roadway or within vegetation along canals and levees at 100 m spacing. Chicken eggs (capsaicin treated or not) were placed in hanging bait cages inside each live trap. Traps were checked daily and reset if needed. The egg was replaced if broken or missing or after 3 days if still present. Camera traps were aimed at baited live traps to document both tegus and non-target species (Moultrie Model # MCG-13331, MCG-13273) at sites A, J, and D. Doing so allowed us to monitor if traps were visited by tegus but were not catching them, and to identify non-target species that potentially decreased trapping efficiency. Camera trap data and batteries were exchanged weekly and photos analyzed within 1-3 weeks. To reduce the number of recaptures of the same individual in camera trap data, once a species was recorded near a trap in the frame, no additional individual of that species was recorded for 10 minutes following its first observation.



Figure 1. Map of study sites for experiments 1 and 2 in Georgia, and experiment 3 in Florida. Experiment 1 was carried out at site A and J (1.1 km from site A) in Tattnall County that contain habituated non-target species. Site A had numerous tegu captures and most of the trapping effort. Site J was a potential corridor for movement of tegus. Non-target species were habituated to both traps and chicken eggs as bait by the start of trapping on 17 March 2020. Experiment 2 was conducted at site D was in Candler County, 45.4 km north of site A. Site D did not have tegus nor were mammals habituated to traps with eggs as bait. Experiment 3 was carried out at site F near the eastern border of Everglades National Park in southern Florida (not shown), where tegu control efforts have taken place for approximately 10 years (see Udell et al. 2022).

Experiment 1: Trap disturbance with habituated non-target species (Georgia, sites A and J)

This experiment quantified trap disturbance rates and tested if non-target species habituated to traps baited with chicken eggs would be deterred by eggs treated with capsaicin. Trapping for tegus occurred at two adjacent sites: site A and site J, which were 1.1 km apart (Fig. 1). Trapping commenced at site J simultaneously because this site was a potential corridor away from site A and to a lowland stream corridor (Rocky Creek) that tegus may also use. We conducted experiment 1 at site A from 5 to 25 October 2020 in which control or capsaicin-treated eggs were used as bait. Site J continued to use untreated eggs during this period. Traps at both sites were closed for 4 days prior to re-opening on 5 October to begin the experiment. This experiment was conducted in October because tegus activity had ceased and thus the sampling was focused on mammalian responses to treated bait.

Treatment and control chicken eggs were lightly cracked by tapping with a hard object and a 16 ga needle was used to withdraw 10 mL albumin from each egg. Treatment eggs were injected with 10mL of a vegetable oil solution containing 0.55 g capsaicin powder per mL. Control eggs were injected with 10mL vegetable oil. Eggs were injected with treatment or control solution on the morning of deployment. Within the 33-trap array at site A, 17 traps received control eggs, and 16 traps received capsaicin treated eggs. Treatment designations were chosen using a random number generator to order traps. At the adjacent site J, 20 traps received untreated eggs (without treatment or control solution) as they had for the rest of the season. Eggs were replaced after being out for 72hrs (3 full days) if they had not been broken or eaten.

Experiment 2: Trap disturbance at novel site without habituated mammals (Georgia, site D)

This experiment tested if naïve mammals that were unexposed to live traps, eggs, or capsaicin-treated eggs would disturb traps as mammals did at site A. Site D was 45.4 km northeast from site A and site J. Opossums, raccoons, and similar mammalian mesopredators are present at site D (M. Cawthorn, C. R. Chandler, Georgia Southern University, verbal pers. comm. 2 April 2021). One trap array (20 traps) at site D received only untreated (control) eggs as bait, while an adjacent trap array (20 traps 70+ m south) was designated to receive only capsaicin treated eggs. A small wash ran through the middle of each site, and grid arrays were laid out the same, as they were in experiment 1. Traps were opened on 26 April 2021 and closed on 4 June 2021. Eggs in traps were replaced after being out for 72 hrs (3 full days) if they had not been broken or eaten.

In experiment 2, treatment eggs were prepared by brushing on 0.5 mL of a solution of 0.12g capsaicin powder per mL of distilled water. A disposable 1 mL pipette was used to drip the solution on the egg, then eggs were left in the refrigerator overnight to dry. We used this method because 1) it is less time consuming and easier to apply than injecting the eggs as in experiment 1, thereby providing a more feasible management option if effective, and 2) because mammals may be more easily exposed to capsaicin on the surface of the egg rather than inside the egg (i.e., detection does not require breaking the egg).

Experiment 3: Trap success on tegus using capsaicin treated eggs (Florida, site F)

As in experiments 1 and 2, we quantified if tegus were trapped at the same rate using either untreated or capsaicin-treated eggs as bait. Because tegus in Georgia are rarely trapped, this experiment was conducted in a larger, well-established population of tegus in southern Miami-Dade County, Florida, outside Everglades National Park (site F). Trapping efforts have been ongoing in southern Florida since 2012, and multiple organizations together remove hundreds of tegus each year (Harvey et al. 2021). Forty-one small live traps were deployed in a linear array at 100 m spacing in a crossover design. Every other trap was baited with a capsaicin-treated chicken egg (n = 20) or with chicken eggs (n = 21) from 8 July - 23 July 2021 (10 trap nights per trap). The treatments were reversed from 26 July -
13 August 2021 (12 trap nights per trap), and again from 16 August – 18 August 2021 (2 trap nights per trap). The cumulative trap night count was started over when control and treatment groups were switched. Consequently, each trap experienced each treatment for 12 full trap nights. The traps were opened every Monday, closed every Friday, and checked each intervening day. Baits were replaced each Monday or more often as needed.

Statistical analyses

To analyze the data, we calculated the proportion of disturbed traps for each day grouped by treatment (3 levels for experiment 1: control [site A], capsaicin-treated [site A], untreated [site]]; 2 levels for experiment 2: control [site D], capsaicin treated [site D]; 2 levels for experiment 3: control [site F], capsaicin-treated [site F]). For experiment 1, we additionally calculated the proportion of disturbed traps within each treatment by trap type (small modified, medium modified, or medium unmodified). For each day, a trap was counted as disturbed if there was any evidence of an animal physically interacting with the trap within a 24hr trapping period. Specifically, we counted a trap as disturbed if the trap had been falsely tripped (closed with no capture), if it had been flipped, if the bait was broken or missing, or if an animal was captured. We recorded a trap as undisturbed if the trap was found open and with bait intact after a 24hr period. To estimate trapping effort, we recorded an undisturbed 24hr trap night as 1, and a disturbed trap night as 0.5 under the assumption that a disturbed trap was fully available to capture an animal for an unknown proportion of the trapping period (we assume half the period as an estimate: Nelson and Clark 1973). We recorded a trap night for broken or missing traps as 0. We then calculated the cumulative trap nights for each day to be included as a covariate in each model. We did this to account for the effect of increased exposure to treatments on animal behavior (e.g., a raccoon may learn to avoid capsaicin treated traps over time).

We fit generalized-linear-models with a binomial distribution for each experiment using the built-in 'glm' function in R (version 4.1.1) software (R Core Team 2021). For experiment 1, we fit the daily proportion of disturbance predicted by treatment, trap type, cumulative trap night, an interaction between treatment and trap type, and an interaction between treatment and cumulative trap night. For experiment 2, we fit daily proportion of disturbance predicted by treatment, cumulative trap night, and an interaction between treatment and cumulative trap night. For experiment 3, we fit daily proportion of disturbance predicted by treatment, cumulative trap night, and an interaction between treatment and cumulative trap night. For experiment 3, we fit daily proportion of disturbance predicted by treatment, cumulative trap night, and an interaction between treatment and cumulative trap night. For experiment 3, we additionally fit the daily proportion of captured tegus predicted by treatment, cumulative trap night, and an interaction between treatment and cumulative trap night. We then tested for significance of explanatory variables using likelihood-ratio χ^2 tests using the 'car' package in R (version 3.1-2; Fox and Weisberg 2019). We considered effects significant if the probability of the observed χ^2_{DF} value was less than 0.05.

Data resources

Data analyzed in the study are available in McBrayer et al. 2023.

Results

Feeding trials

Each tegu fed freely in five trials where non-capsaicin-coated food items were presented ($\bar{x} = 12.8$ items ± 7.5 SD). During nine experimental feeding trails using capsaicin treated food, tegus ate both capsaicin and control food items (control: $\bar{x} = 5.78 \pm 4.84$ SD; capsaicin: $\bar{x} = 3.89 \pm 2.93$ SD). Both within and across trials, tegus ate capsaicin treated food and did not learn to refuse it. Yet, tegus ate more of the untreated food than capsaicin treated food (McBrayer et al. 2023).

Documentation of tegus and non-target species at the sites (A, J, and D) in Georgia

During the sampling period, eight tegus were observed on the cameras visiting traps. During our sampling period, at live traps without corresponding camera traps, two tegus were captured at site A.

Mammals comprised the majority of non-target observations in Georgia at sites A, J, and D (Table 1, Suppl. material 1: table S1), and the rate of trap disturbance varied spatially (i.e., among sites) and temporally (i.e., within sites as the experiment progressed). For context, one Opossum, one raccoon and two birds were documented in the first four days of camera trapping at site A, and trap visitation and disturbance by non-targets rapidly increased from this point forward (see below). Between 22 April 2020 to 03 October 2020, camera traps documented 1445 raccoons (Procyon lotor), 554 opossums (Didelphis virginiana), and 212 armadillos (Dasypus novemcinctus) visiting or disturbing traps. In total, we gathered 3498 observations of 33 species (or taxa) via camera traps (Suppl. material 1: table S1). Most importantly, camera traps in 2020 documented that raccoons, in particular, disturb traps by trying to enter the trap, reaching inside (but not entering it), shaking and climbing on the trap, throwing it, etc. These actions caused the trip plate to trigger the closure of the trap door, whereby neither the mammal nor a tegu could be trapped. Although other taxa occasionally disturbed traps set for tegus (e.g., diurnal bird species, turtles, rodents; Haro et al. 2020 table 5), these taxa were frequently trapped, and thus easily identified, or were observed infrequently on game cameras.

Experiment I: Trap disturbance with habituated non-target species (Georgia sites A and J) $\!\!\!$

Before beginning experiment 1, trap disturbance rose quickly at sites A and J such that by mid-July 2020, resident mammals were habituated to traps baited with chicken eggs and it was not uncommon to have 100% of the traps disturbed each night. The focal site (site A) experienced a relatively high amount of trap disturbance (>0.8) immediately, while site J experienced lower daily disturbance until 1250 cumulative trap **Table 1.** Summary of live tegu trap effort and success at four locations under three experiments (Exp.). In Georgia (GA), tegus were only captured at site A, while mammals were the principal taxon captured in live traps both at sites A, J, and D. Site J was 1.1km from site A (both in Tattnall County) and a potential movement corridor for tegus. Sites A and J contained non-target species habituated to live traps. Tegus were not present at site D (Candler County), and mammals at site D were not habituated to either live traps or bait (eggs). In Florida (Miami-Dade County), site F has high trapping success for tegus and lower rates of disturbance from mammals. Capsaicin treated bait failed to deter mammals or other taxa from disturbing eggs inside traps (McBrayer et al. 2023).

Site	Date Range	Treatment	Total Trap-	Total Animals	Total Tegus	Percent	Percent Non-
			nights	Caught	Caught	Mammals	mammals
А	Mar 17 – Oct 1, 2020	Untreated, Exp. 1	2746.0	26	2	73	27
J	Mar 18 – Sep 30, 2020	Untreated, Exp. 1	2103.5	32	0	59	41
А	Oct 5 – Oct 25, 2020	Capsaicin, Exp. 1	176.5	0	0	0	0
А	Oct 5 – Oct 25, 2020	Control, Exp. 1	192.0	3	0	100	0
J	Oct 5 – Oct 25, 2020	Untreated, Exp. 1	223.5	5	0	100	0
D	Apr 26 – Jun 4, 2021	Capsaicin, Exp. 2	511.5	4	0	100	0
D	Apr 26 – Jun 4, 2021	Control, Exp. 2	496.0	4	0	50	50
F	Jul 8 – Aug 18, 2021	Capsaicin, Exp. 3	419.0	40	31	17	83
F	Jul 8 – Aug 18, 2021	Control, Exp. 3	417.5	49	42	8	92

nights when disturbance rose to about 0.8 (Fig. 2A). Once trap disturbance rose to 0.80, it seldom abated for the remainder of the season (Fig. 2B). From May to August 2020, non-target species, principally consisting of raccoons and opossums, were verified with game cameras at site A.

Between 5 and 25 October 2020, traps at site A were baited with either capsaicin treated eggs, or control eggs. During this time, the proportion of disturbed traps did not change depending on trap type (GLM: $\chi_2^2 = 0.972$, P = 0.615), nor with cumulative trap nights (GLM: $\chi_1^2 = 0.026$, P = 0.873; Fig. 3). Traps using capsaicin treated eggs experienced the same level of disturbance as those treated with control or untreated eggs (GLM: $\chi_2^2 = 0.156$, P = 0.925). Thus, eggs treated with capsaicin did not result in lower trap disturbance by non-target species habituated to bait reward.

Experiment 2: Trap disturbance at novel site without habituated mammals (Georgia site D)

Non-target mammal species at site D became habituated to traps and eggs rapidly (see Suppl. material 1: table S2), just as they did at site A. Here, trap disturbance rose to > 0.80 in roughly 175 trap nights (Fig. 4). A significant relationship was found between cumulative traps nights and proportion of disturbed traps (GLM: $\chi^2_1 = 16.075$, P < 0.001). For a one unit increase in trap night, the odds of trap disturbance versus no disturbance increased by a factor of 1.008 (95% CI [1.004, 1.012]). This effect of cumulative trap night did not differ between treatments (GLM: $\chi^2_1 = 0.006$, P = 0.936). There was no significant effect of bait treatment on the proportion of disturbed traps (GLM: $\chi^2_1 = 0.439$, P < 0.508).



Figure 2. A proportion of traps disturbed early in the season (17 March to 15 June 2020) during experiment 1 at sites A and J in Georgia (McBrayer et al. 2023). Note that non-target species disturbed > 0.80 of traps within roughly 800 traps nights at site A, and 1250 trap nights at site J. **B** proportion of traps disturbed by non-target species across the entire season (17 March to 25 October 2020) in experiment 1 at sites A and J. Widespread trap disturbance continued for the remainder of the season. Black (site A) represents traps using control and capsaicin treated bait, while grey represents a site 1.1 km from site A (site J) that received only control eggs.



Figure 3. In experiment 1, the proportion of disturbed traps between 05 to 25 October 2020. Site A showed a decline by treatment type (capsaicin treated bait *vs.* control and untreated bait at two nearby sites A and J) over time in the direction expected, but the trend was not statistically significant. At each of the three sites, capsaicin did not significantly lower disturbance rates in this experiment (McBrayer et al. 2023).



Figure 4. Trap disturbance in experiment 2 as a function of cumulative trap nights, where non-target species were not habituated to traps or bait (site D; 45.4 km from site A). Capsaicin-coated eggs did not significantly lower disturbance rates in this experiment (McBrayer et al. 2023).

Experiment 3: Trap success on tegus using capsaicin treated eggs (Florida site F)

In southern Florida (Miami-Dade, site F), 31 tegus were captured using capsaicintreated eggs, whereas 42 tegus were captured using eggs without capsaicin; tegu capture rates did not significantly differ between bait type (GLM: $\chi_1^2 = 0.005$, P = 0.941). There was no effect of cumulative trap night on tegu capture rates (GLM: $\chi_1^2 = 0.012$, P = 0.911; Fig. 5). Though daily disturbance rates were relatively low at this site (mean = 0.25), there was not a significant difference in disturbance rates according to bait type (GLM: $\chi_1^2 = 0.002$, P = 0.966). Additionally, disturbance rates did not change as cumulative trap nights increased (GLM: $\chi_1^2 = 0.0002$, P = 0.993).

Discussion

Capsaicin-treated food did not deter three captive tegus from feeding in lab trials. Similarly, there was not a significant difference in tegu captures using capsaicin-treated vs. untreated baits at site F (Florida), though fewer tegus were trapped using capsaicin-treated baits. Together, these two experiments suggest tegus are likely tolerant of capsaicin. Although a promising finding, capsaicin-treated baits did not reduce the trap disturbance by non-target species at the sites in Georgia, where disturbance rates reach \geq 80% and are a significant impediment to trapping tegus. Non-target mammal species rapidly caused high trap-disturbance rates at site A where non-targets were likely habituated to traps baited with chicken eggs (experiment 1, Fig. 2). We suggest that non-targets became habituated to traps because (a) trapping occurred at this site in 2019 and disturbance was high, and (b) the rate of disturbance increased rapidly and was unchanged throughout the season, and (c) similar patterns were observed by non-targets without exposure to egg-baited traps or capsaicin (experiment 2, Fig. 4). Hence, rapid habituation to an egg reward may have contributed to the lack of any statistically significant effect of capsaicin to decrease trap disturbance.

Our results show how non-target disturbance varies spatially (within GA and GA to FL), which underscores how management strategies may also vary to effectively remove invasive species (Table 1). Daily disturbance rates in Florida (site F) are likely low for two reasons: one, the abundance of meso-mammals has significantly declined in association with invasive Burmese pythons and raccoons are now uncommon (Dorcas et al. 2012); and two, small, not medium, traps were used in Florida such that adult meso-mammals may be less likely to be trapped (if present). Adult tegus were trapped in medium traps in 2019 at site A (Haro et al. 2020). In 2020, small and medium traps were used to test if tegus would enter larger (medium) and/or smaller (small) traps. Raccoon and possum disturbed both trap sizes in Georgia, yet astonishingly few raccoons were actually trapped, instead simply disturbing the trap without getting captured. At site F, small rodents caused disturbance, but at low rates, possibly because they may be less interested in the bait and could be exploring traps as a novel aspect of the habitat. The somewhat trivial rate of trap disturbance in the Florida population does not warrant use of a deterrent since large numbers of tegus are caught annually



Figure 5. Tegu capture rates (**A**) and trap disturbance rates (**B**) during experiment 3 at site F. Neither tegu capture rates nor trap disturbance rates differed between bait treatment types in experiment 3. Non-target species did not disturb traps as much as at sites in southeast Georgia. Trap disturbance was not significantly associated with cumulative trap nights. Also, capsaicin treated bait did not have a significant effect on tegu capture rate (McBrayer et al. 2023). Trend lines added for visualization only.

(Harvey et al. 2021). Yet for the incipient tegu population in Georgia, trap disturbance is a more urgent issue because meso-mammals are abundant (Suppl. material 1: table S1), and EDRR efforts to remove tegus are compromised by trap disturbance.

Limited published data exist on reducing non-target trap captures. Standard suggestions include alternative capture methods or trap types, trapping timing (season and time of day), bait, and trap placement (Peitz et al. 2001). Traps could be physically modified to specifically reduce the possibility of non-target disturbance. To that end, we staked our traps down with 0.4m long stakes of rebar at the Georgia sites, however doing so failed to decrease trap disturbance. Instead, future efforts might try covering the trap and its entrance with corrugated pipe (or similar) such that non-targets cannot reach into the live trap (Roden-Reynolds et al. 2018).

Another approach to mitigate non-target species disturbance could be to open traps based on target vs. non-target species behavior or activity time. Raccoons are known to move between major and minor feeding areas during the night (Lotze and Anderson 1979). Understanding non-target species movements could help place traps in non-foraging areas or areas with less movement by non-target species. Camera traps revealed low rates of diurnal species entering or disturbing traps (raccoons and birds were most common), yet reptiles and amphibians were rare (Suppl. material 1: table S1; McBrayer et al. 2023). For smaller trap arrays for diurnal target species, closing traps or removing bait from traps near sunset, and replacing bait again near sunrise, may present a feasible method to reduce trap disturbance by nocturnal species. Yet, removing bait or closing traps at night would be labor intensive and costly. At site F (Florida), the use of capsaicin-treated bait increased the time to set or reset traps which also increased the time to run trap lines. An effective deterrent for non-target species would be widely available and inexpensive, simple to introduce and quickly implement, and be highly deterrent if it is to be adopted on a large scale (e.g., Lacey et al. 2015).

Chemical (gustatory) and olfactory deterrents may continue to show promise (e.g., Conover, 1989), even though capsaicin was not effective here. Coyote urine is not an effective deterrent for raccoons or opossums (Yocom-Russel and Verble 2020). However, 2% anthraquinone was shown to repel raccoon feeding on corn by 71%. Anthraquinone is a naturally occurring compound and has been used to repel rodents, rabbits (Werner et al. 2016), and pigs (Snow et al. 2021), though it also repels birds (DeLiberto and Werner 2016), suggesting it may have a similar repellent effect on tegus and other reptiles. Likewise, conditioned food aversions may have the potential to deter some species (reviewed in Snijders et al. 2021). In a captive trial, raccoons developed a taste aversion to oral estrogen concealed in an egg (Dueser et al. 2018). Hence, conditioned food aversion trials to deter problematic species like raccoons could be conducted prior to invasive species trapping.

Conclusion

Tegus represent a threat to native species once established (Klug et al. 2015; Mazzotti et al. 2015; Haro et al. 2020). Thus, future experiments to understand how non-target species might be efficiently deterred from disturbing live traps for this invasive species may be useful for control. Here we show that raccoons and opossums readily learn to disturb live traps set for tegus and are not deterred by bait coated with capsaicin. Removal of incipient populations of tegus could be fraught with difficulty if methods to reduce trap disturbance are not identified and deployed.

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Supplementary material I

Species lists of non-target species disturbing traps set for tegu lizards

Authors: Lance D. McBrayer, Daniel Haro, Michael Brennan, Bryan G. Falk, Amy A. Yackel Adams

Data type: Occurence data

- Explanation note: Species observed at one site during the study and species observed at a second sites about 40 km away.
- 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



Testing a modified version of the EPPO decision-support scheme for release of classical biological control agents of plant pests using Ganaspis cf. brasiliensis and Cleruchoides noackae as case studies

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Abstract

The 6/04 standard of the European and Mediterranean Plant Protection Organisation (EPPO) on the safe use of biological control is a decision-support scheme (DSS) for the import and release of biological control agents in Europe. It was recently developed by the Joint EPPO/International Organisation of Biological Control (IOBC) Panel on Biological Control Agents. The DSS can be used to assess the potential environmental impacts of biological control agents. It is valid for different types of biological control: classical and augmentative biological control of invertebrates, pathogens and weeds. However, the DSS is not yet widely implemented in Europe and, during preliminary trials, it was found that its broad range of usages could lead to some confusion or misunderstandings, as well as requiring unnecessary information in some cases. Thus, the scheme was modified to specifically assess classical biological control against plant pests, i.e. the introduction of exotic natural enemies of plant pests for establishment and long-term control. The new version of the scheme was then tested on two parasitoids that are presently being released in Europe, the figitid Ganaspis cf. brasiliensis against the spotted wing drosophila Drosophila suzukii and the mymarid Cleruchoides noackae against the Eucalyptus bronze bug Thaumastocoris peregrinus. Both parasitoids were successfully assessed with the new version of the DSS. No major issues were encountered during the assessments and most questions were answered with low levels of uncertainty. Both assessments concluded that the parasitoids were safe to release in the impact assessment areas, with positive impacts exceeding negative impacts. Suggestions for potential improvements are provided.

Keywords

bronze bug, Drosophila suzukii, importation, invasive species, Thaumastocoris peregrinus

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Introduction

The number of invasive plant pests, in particular herbivorous insects and plant pathogens, is increasing dramatically around the world, despite improved quarantine measures and border security (Santini et al. 2013; Roques et al. 2016; Seebens et al. 2017). This increase is largely due to the exponential growth of the transcontinental trade of fruits, vegetables, live plants and other plant products, such as wood packaging material, seeds and cut flowers, on which plant pests are travelling inconspicuously (Kenis et al. 2007; Santini et al. 2018). Once established, invasive plant pests are difficult to manage because of their uncontrolled development in regions where natural enemies are lacking and host plants have not developed resistance mechanisms against them (Desurmont and Pearse 2014; Garnas et al. 2016; Lovett et al. 2016; McLaughlin and Dearden 2019).

One method for long-term management of invasive plant pests is classical biological control (CBC), i.e. the introduction of natural enemies of exotic origin to control invasive pests, aiming at permanent control of the pest (Van Driesche and Bellows 1996). CBC has been implemented since the 19th century, mostly against invasive insects using parasitoids and predators. Over 700 insect pests have been targeted, with significant successes (Cock et al. 2015, 2016; Kenis et al. 2017). However, in some cases, negative effects have been observed on non-target species, especially when polyphagous parasitoids or predators were used (Hajek et al. 2016). Therefore, nowadays, the introduction of CBC agents usually follows strict procedures, including a rigorous assessment of their potential non-target effects (Van Driesche and Hoddle 2016). Even so, regulatory frameworks and procedures for the assessment and release of CBC agents vary significantly amongst countries and continents, according to their own legal and societal context and their history of using biological control practices (Afonso et al., in press).

In Europe, the use of classical biological control is hampered by the heterogeneity of national legislation (when existing) and practices and by the lack of common regulations. The European and Mediterranean Plant Protection Organisation (EPPO) has recently published the standard PM 6/04 on the safe use of biological control, presented as a decision-support scheme (DSS) for the import and release of biological control agents of plant pests (EPPO 2018). It was written mainly for decision-makers and assessors of biological control applications to harmonise assessment procedures within EPPO countries. It provides detailed instructions for environmental impact assessment for biological control agents of plant pests for augmentative biological control (ABC) and classical biological control. Although its title states it is for biological control agents of plant pests, it can also be applied to biological control agents of weeds.

However, the EPPO PM 6/04 standard is not yet widely implemented in Europe. In this paper, we tested it to assess the potential as well as safety of two exotic parasitoids, *Ganaspis* cf. *brasiliensis* (Ihering) (Hym., Figitidae) and *Cleruchoides noackae* L in and Huber (Hym., Mymaridae), for release against *Drosophila suzukii* Matsumura (Dipt., Drosophilidae) and *Thaumastocoris peregrinus* Carpintero and Dellapé (Hem., Thaumastocoridae), respectively. In preliminary trials, we noticed ambiguities due to the fact that the DDS was made to cover both augmentative and classical biological control agents (including native species) in addition to invertebrate and weed biological control. Therefore, we modified the scheme and retained only questions relevant for CBC against invertebrates using exotic natural enemies.

Description of the original EPPO standard PM 6/4

The EPPO standard PM 6/04 "Decision-support scheme for import and release of biological control agents of plant pests" (EPPO 2018) was prepared by the Joint EPPO/ IOBC Panel on Biological Control Agents. It follows the recommendations of the International Standard for Phytosanitary measures 3 (ISPM 3) "Guidelines for the export, shipment, import and release of biological control agents and other beneficial organisms" (FAO 2017) and complements the earlier EPPO Standard PM 6/2 "Import and release of non-indigenous biological control agents" (EPPO 2014) by converting the guidelines into a question-based decision-support scheme.

The DSS is based on a sequence of questions that aim at deciding whether the introduction of a biological control agent (BCA) could cause unwanted environmental impacts and to compare the likelihood and impact of such negative effects with potential positive environmental effects. The DSS is a comprehensive document and rather unique amongst protocols and standards to assess the potential environmental impacts of biological control agents. To the best of our knowledge, no other DSS exist for environmental impact assessments (EIA) of biological control agents. It is valid for different types of biological control: classical and augmentative biological control of invertebrates, pathogens and weeds.

The document consists of two main parts: (I) an express scheme which may produce a rapid result and (II) a comprehensive scheme for certain cases of biological control, for which the express scheme does not lead to sufficiently clear recommendations. Within part I, the following steps are included: Step 1 Initiation; Step 2 BCA categorisation; Step 3 Impact assessment (four questions); Step 4 Decision taking. Part II consists of: Step 1 Pre-assessment (18 questions); Step 2 Assessment of probability of establishment (20 questions); Step 3 Assessment of probability of spread (three questions); Step 4 Assessment of potential environmental consequences (37 questions); Step 5 Recording the degree and types of uncertainty (one part); Step 6 Conclusion of the EIA. The latter is subdivided into the categories: establishment; spread; environmental impact; overall conclusion.

Case study I: Drosophila suzukii and its parasitoid Ganaspis cf. brasiliensis

Drosophila suzukii is an East Asian fruit fly that is particularly damaging for small fruits such as cherry, strawberry, raspberry, blueberry and blackberry. In contrast to most other drosophilids, females of *D. suzukii* have a strongly serrated ovipositor and can lay eggs

through the skin of mature, undamaged fruits (Asplen et al. 2015). It was first found in Europe and North America in 2008 and it is now present in most European countries, as well as in numerous regions of North and South America, western Asia and Africa (Rossi-Stacconi 2022). *Drosophila suzukii* is particularly difficult to control in invaded regions (Mazzi et al. 2017; Santoiemma et al. 2020; Tait et al. 2021). Current control methods are restrictive and expensive, especially because *D. suzukii* has many generations per year in a very large number of cultivated and wild fruits (Kenis et al. 2016). As a result, crops are constantly invaded from the surroundings. Against this type of invasive pest, large-scale control strategies, such as CBC, are recommended, to lower pest populations both in cultivated and wild habitats (Haye et al. 2016; Wang et al. 2020).

Extensive surveys for parasitoids of *D. suzukii* have been conducted in the area of origin of the pest, viz. Japan, China and South Korea (Kasuya et al. 2013; Daane et al. 2016; Girod et al. 2018a; Matsuura et al. 2018; Fang et al. 2019; Giorgini et al. 2019; Kimura and Mitsui 2020). In most surveys, the figitid wasp Ganaspis brasiliensis was the most abundant parasitoid. Subsequent studies in the laboratory also showed that this species was more specific than other common parasitoids of D. suzukii, such as Asobara japonica and Leptopilina japonica (Girod et al. 2018b; Giorgini et al. 2019; Daane et al. 2021). However, they also showed marked differences in host specificity amongst populations of G. brasiliensis (Girod et al. 2018b; Seehausen et al. 2020). Previous molecular studies in Japan by Nomano et al. (2017) had already shown that G. brasiliensis was composed of five genetic groups (corresponding to biotypes or possibly cryptic species), with different host preferences and geographic distributions. Surveys in Asia and subsequent molecular analyses revealed that at least two or three genetically distinct groups (G1, G3 and possibly G4) attack D. suzukii (Giorgini et al. 2019; Seehausen et al. 2020). Under laboratory conditions, one genetic group (G3-4) readily parasitised some closely-related Drosophila species, such as D. melanogaster and D. simulans regardless of their food source. In contrast, the other genetic group (G1) was almost entirely specific to larvae feeding in ripening fruits (Seehausen et al. 2020). Further studies showed marked genetic differences between those populations (Seehausen et al. 2020) and their expressed proteins (Reeve and Seehausen 2019). In addition, these two groups did not interbreed and it is plausible that these are two cryptic species. The discovery that G. cf. brasiliensis is likely a species complex of at least two congeneric species and that one of them can clearly be associated with a higher habitat specificity to fresh fruits, shows the suitability of G. cf. brasiliensis G1 to control D. suzukii. This allows research to focus on that species to assess its potential as a classical biological control agent. The difference in natural habitat preference between immature D. suzukii developing in fresh ripe fruits and other frugivorous Drosophila species in decomposing fruits supports the hypothesis that G. cf. brasiliensis G1 is hostspecific. This specialisation would then support the conclusion that G. cf. brasiliensis G1 wasps will only parasitise D. suzukii in its invaded range.

In 2021, large-arena field cage releases of *G*. cf. *brasiliensis* G1 conducted in Switzerland confirmed that this species has a very high preference for *D*. *suzukii* in fresh fruits (Seehausen et al. 2022). At the same time, *G*. cf. *brasiliensis* G1 was found to be adventively present in western Canada (Abram et al. 2020) and the western USA (Beers et al. 2022).

The modified EPPO DSS for *G*. cf. *brasiliensis* G1 included an impact assessment area (IAA) comprising all European countries west of Russia (excluding EU and UK Overseas Countries and Territories). For the purpose of testing the DSS, the risk assessment was carried out, based on the assumption that no release has been made in Europe at the time of the assessment, although *G*. cf. *brasiliensis* G1 was released in 2021 in Italy after a risk assessment was conducted at the national level (Lisi et al. 2022; Fellin et al. 2023).

Case study 2: Thaumastocoris peregrinus and its parasitoid Cleruchoides noackae

The Eucalyptus bronze bug *Thaumastocoris peregrinus* Carpintero and Dellapé, (Hem., Thaumastocoridae) is native to Australia where it is distributed across several climatic regions (Nadel et al. 2012). This hemipteran is a sap sucker that feeds on the foliage of a broad range of *Eucalyptus* species. Its feeding activity causes necrosis and discolouration of the tree canopy. Attacked leaves exhibit a characteristic bronze colour, followed by drying. The intensive damage in the canopy results in tree growth losses and weakens the trees. Both in its native and invaded range, the bronze bug is found in forest plantations as well as on ornamental trees in urban areas, such as in streets and gardens (Nadel et al. 2012; Laudonia and Sasso 2012; Garcia et al. 2013). Extensive damage has been reported in the countries where this species is established, with significant economic losses. For example, the loss in wood production caused by *T. peregrinus* was estimated to exceed US\$ 380 per hectare in Brazil (Junqueira et al. 2018). Many ecosystem services provided by *Eucalyptus* trees are also disrupted by the *Eucalyptus* defoliation (Branco et al. 2015).

The bronze bug was first found outside Australia in South Africa in 2003 (Jacobs and Neser 2005) and in Argentina in 2005 (Noack and Coviella 2006). These two separate introductions correspond to two different Cytochrome Oxidase subunit 1 (COI) haplotypes (Machado et al. 2020), but probably both originate from a population in Sydney (Montagu et al. 2020). From Argentina, the bronze bug spread rapidly to nearby countries in South America and, similarly, from South Africa, it spread to neighbouring African countries (Hurley et al. 2011). In Europe, the species was first found in Italy in 2011 (Laudonia and Sasso 2012) and later in Portugal (Garcia et al. 2013) and Spain (Nascimento-Machado et al. 2019). European populations are the same haplotype as that present in South America (Machado et al. 2020). Molecular data support the hypothesis that the bronze bug was introduced from South America to Europe and then spread to Israel (Montagu et al. 2020).

Insecticides such as imidacloprid, thiamethoxam and acephate were tested and shown to be effective against the bronze bug (Noack et al. 2009; Wilcken et al. 2019). Biopesticides *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Cordycipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Clavicipitaceae) also showed good results (Wilcken et al. 2019). However, insecticides are not economically nor ecologically sustainable, especially as a long-term strategy and their application in forest plantations and urban areas is highly restricted by national regulations. The search for biopesticides provided promising results with *B. bassiana* (Corallo et al. 2019). However, there is a

high variability amongst strains and the biopesticide's efficiency is highly dependent on environmental conditions, such as temperature and humidity. Moreover, the need for continuous applications renders this management strategy costly. Selection for host resistance was not considered a viable management option, due to the broad host range of *T. peregrinus* (Nadel and Noack 2012). Biological control using the egg parasitoid *Cleruchoides noackae* Lin and Huber (Hym., Mymaridae) is currently the main control strategy in the invaded areas with effective and sustainable long-lasting results.

Cleruchoides noackae is a tiny wasp, < 0.5 mm in length, which was described by Lin et al. (2007) from parasitised eggs of *T. peregrinus* collected in Australia. The species can be unambiguously identified by morphological traits. Molecular analysis, using COI confirmed that *C. noackae* is widespread in Australia (Nadel et al. 2012).

The host range of *C. noackae* consists of species within the Thaumastocorinae subfamily, in particular, in the genera *Thaumastocoris* and *Baclozygum* (Lin et al. 2007; Cross 2009). The family Thaumastocoridae is native to Australasian regions and there are no species native to Europe. Therefore, non-target parasitism is unlikely in the introduced range. An unpublished study in Portugal (CA and MB) used no-choice tests to assess potential use of 19 non-target host species within eight Hemiptera families (Anthocoridae, Miridae, Tingidae, Coreidae, Pentatomidae, Pyrrhocoridae, Rhopalidae and Scutelleridae). Parasitism occurred only on *T. peregrinus*, suggesting that *C. noackae* will exclusively attack only the target host in Europe.

Cleruchoides noackae was released and became established in South Africa, South America (Chile, Brazil and Uruguay) (Nadel and Noack 2012; Barbosa et al. 2017) and Israel (Mendel and Protasov 2019). In a post-release study conducted in Brazil, Barbosa et al. (2017) showed that one year after the release, the parasitoid could be found up to 10 km from the release site. Given the small size of *C. noackae*, its capacity for active dispersal is possibly limited. Still, there is a high possibility of natural dispersal by wind.

Due to the specialised trophic relationships amongst *C. noackae*, *T. peregrinus* and *Eucalyptus* spp., in which all are non-native to Europe, indirect effects on non-target species are highly unlikely, as well as other negative environmental effects in native flora and fauna.

Release of *C. noackae* in Portugal was authorised in 2021 by the Portuguese National authorities, ICNF – Instituto de Conservação da Natureza e Florestas with the condition of a no release buffer zone of 20 km on the border with Spain. Initial releases were made in October 2021 in Central Portugal and post-release monitoring data are not yet available. *Cleruchoides noackae* is not present in other European countries where it could have a beneficial impact.

The modified EPPO DSS was tested for release of *C. noackae* in southern Europe. The IAA includes all European countries where *Eucalyptus* trees are planted and used for production or amenity objectives. Once again, for the purpose of testing the DSS, the risk assessment was carried out with the assumption that no release has been made in Europe at the time of the assessment.

Critical evaluation of the EPPO DSS

The DSS includes questions about any potential earlier assessments (section 1.2 in the Express assessment and 1.04 in the Full assessment), which may save resources. If the BCA, or a very similar organism (e.g. another population of the same species), may have been subjected to an EIA process before, nationally or internationally, this may partly or entirely replace the need for a new assessment. These questions could be enhanced by requesting information about previous releases of the same BCA, for example, in similar regions/conditions (if yes, how long ago?) and what was the outcome in terms of establishment, impact on target species and non-target species? For both case studies, *G. cf. brasiliensis* and *C. noackae*, this would be necessary and useful information to render the decision process for or against releases in additional countries more efficient. For example, information of the outcome of prior releases of *C. noackae* in South Africa, South America and Israel were instrumental for the release application in Portugal. Similarly, for the *G. cf. brasiliensis* release application in Switzerland, results from studies on releases or adventive distributions in North America and Italy were considered important information for the risk assessment.

The DSS provides the opportunity to include undescribed cryptic species as potential BCAs, as it includes consideration of taxonomic levels higher or lower than species, including those characterised using molecular methods (Step 2 BCA categorisation of the Express assessment and step 1.02 of the Full assessment). This is important in high priority cases like the CBC of *Drosophila suzukii*, where only one genetic group of the parasitoid *G.* cf. *brasiliensis* (a probable cryptic species) is specific to *D. suzukii* larvae feeding in fresh fruits.

The DSS starts with the Express assessment for invertebrates and pathogens whereas, for weed biocontrol, it is recommended to start directly with the Full assessment. This is based on the higher risk of weed BCAs becoming pests of important crops. While the Express assessment may be convenient in some cases and save resources such as time and money, especially for augmentative BCA, few countries, if any, in the EPPO region would allow an Express assessment for new CBC agents of plant pests. This is especially the case in countries like France, Israel, Italy or Switzerland, where national authorities require comprehensive risk assessments for the release of any nonnative species. Therefore, any new CBC, whether it is introduced against weeds or plant pests, should only be evaluated using the Full assessment.

The sections about agent establishment (Step 2) and spread (Step 3) in the Full assessment are very detailed and comprehensive. While for the assessment of agent establishment, a distinction between different types of biological control (ABC vs. CBC) is possible, this is not the case for the assessment of spread. In ABC, agents are released in the field or in greenhouses to augment the numbers of a species that are native or already established. In the case of ABC for protected crops, an agent is regularly released for temporary control. Establishment in the wild and spread of the released populations of an agent are, in most cases, undesired in ABC, because the aim is to minimise unwanted effects on the environment and non-target species. In contrast, CBC is the intentional introduction of a species into an area where it is not indigenous and, to be successful, it must establish and spread on its own after release. Therefore, for CBC, the section dealing with establishment and spread of a BCA is relevant to assess the feasibility, efficiency and economic risk of a CBC programme, but not for an EIA because the establishment and spread to all areas where the host/prey is present has to be assumed. However, this means that the EIA of CBC agents must very thoroughly assess possible non-target impacts (especially including host specificity) in the entire geographic area to which the impact assessment applies. In contrast, ABC is applied in a defined place such as a cropland and it frequently involves generalist agents. The spread of released BCAs to areas outside the IAA can, therefore, have a detrimental effect on native species and their populations. In the current DSS, the same questions about establishment and dispersal are asked for both ABC and CBC and it remains unclear from the document if the answer is actually a benefit (e.g. high probability of spread and establishment for CBC agents) or a risk (e.g. high probability of spread and establishment for ABC agents). A solution to this problem would be to have a separate DSS for the different types of BC. In a DSS for CBC, many questions about establishment and dispersal would then not be necessary and could be simplified into just a few questions to ensure that establishment is likely. As for the spread, the questions could be totally suppressed, since an established CBC agent will spread anyway, no matter the rapidity of spread. If the natural spread is too slow, the BCA can be distributed through a release programme. Additionally, other questions, for example, whether the BCA is already present or indigenous in the IAA or may cause transient effects, would then become obsolete. Applied to the case studies, a first introduction of the CBC agents into a European country would imply that establishment and spread into neighbouring countries, where the target and its host plant are present and where climate is suitable has to be assumed and thus, the IAA needs to include these countries. In the case of C. noackae, all southern European countries where Eucalyptus trees are planted would be included and, for G. cf. brasiliensis, all European countries.

Several questions are strictly related to CBC of weeds. While it is clearly mentioned in the DSS that such questions have to be answered only in the case of CBC of weeds, these questions needlessly lengthen the scheme under which CBC agents of invertebrates are assessed. Furthermore, the potential impact of weed BC agents (herbivores and plant pathogens) are different from those caused by invertebrate BC agents (parasitoids, predators and entomopathogens), so that a full separation from CBC of weeds and pathogens would allow more precise responses to questions on impact mechanisms, establishment and spread.

One of the clear strengths of the DSS is the consideration of positive environmental impacts of BCA releases. This is a great step forward compared to most processes that solely consider risks. While the selection of the risks and benefits that are included in the evaluation provide an objective baseline, the decision whether the positive impacts exceed the negative ones, remains a subjective expert opinion. In CBC, decisionmaking processes should weigh both the risks and potential benefits. These benefits should include environmental and socio-economic considerations, such as direct and indirect benefits for farmers/producers and the general public. For example, in the case of *G.* cf. *brasiliensis* releases against *D. suzukii*, environmental benefits (e.g. reduction of pesticide use and reduction of infestation of wild fruits), as well as socio-economic benefits (e.g. reduction of costs for protecting crops), would be expected and these reduce the high losses fruit growers have through this invasive species (Burrack 2015; De Ros et al. 2015; Knapp et al. 2020). However, if the goal of the DSS is to be purely an environmental risk-benefit assessment, this latter suggestion might not be applicable.

Another strength of the DSS is the recording of the degree and types of uncertainty (Step 5 in the Full assessment) when it comes to potential environmental consequences. As some aspects of the risks and benefits may be based on subjective expert opinions, limited data or only on available data from closely-related species, the level and type of uncertainty helps to identify weaknesses in the assessment, research needs or even unacceptable risks for approving releases. What is unclear in this step is the definition of the consequences if the uncertainty is high. A probable consequence in the case of high uncertainty could be the requirement to gather more data through literature reviews or additional tests. Thus, there should be clear directions on what steps should be taken if uncertainties are high.

The overall conclusion of the DSS (Step 4 Decision taking in the Express assessment, Step 6 of the Full assessment) should be a clear acceptance or rejection of the application to import and release a BCA. While this is sufficiently clear in the Express assessment through the binary outcome in sections 4.3 (recommendation for releases) and 4.4 (no recommendation for releases), it is not clear in the Full assessment. However, the Full assessment allows the decision-maker to comment on and justify the conclusion in each of the three major sections: establishment, spread and environmental impact.

Modifications of the DSS for classical biological control of plant pests

Based on the considerations above, we have modified the EPPO Standard PM 6/04 (1) Decision-support scheme for import and release of biological control agents of plant pests (EPPO 2018) for specifically assessing the potential for releases of CBC agents of plant pests (Suppl. material 1). Only modifications essential for this specific type of assessment were made. Other changes, in the format and the phrasing, to be precise, have been kept to a minimum. As for the original standard, the decisions are based only on environmental impacts (negative and positive), not on socio-economic considerations.

The main modifications are as follows:

- The express scheme has been deleted.
- All questions specifically related to weed biological control have been deleted.
- All questions are now unambiguously concerned with classical biological control, i.e. the introduction of an exotic BCA (usually parasitoid or predator) against a plant pest, for a permanent establishment of the BCA leading to long-lasting control of the target pest. Some questions have been modified and all questions referring more specifically to augmentative biological control have been suppressed.
- The section on the assessment of probability of spread has been deleted.

The new scheme is presented in Suppl. material 1. The modified DSS now starts with a short pre-assessment (Step 1) to present the BCA and to ensure that it is a priori suitable for classical biological control in an IAA. Step 2 involves assessing the probability of establishment in the IAA. Step 3 consists of an assessment of potential environmental consequences. The degree and types of uncertainty are considered in Step 4 and Step 5 provides the conclusion of the EIA.

Assessment of Ganaspis cf. brasiliensis using the modified DSS

The Full assessment is presented in Suppl. material 2. For the most part, the assessment was straightforward because the parasitoid has been extensively studied. Thus, most questions were answered indicating low levels of uncertainty. The questions for which answers were given with medium or high uncertainty were particularly those on climate suitability and positive environmental impacts that may result from controlling *D. suzukii*. In some cases, answers were more complex due to the data on the parasitoid coming mostly from laboratory rearing and, to a much lower extent, from the field in its native range, whereas hardly any data are available from other regions of introduction. However, typically, baseline information from the field in the native range and from the quarantine laboratory in the area of introduction is used to predict the potential impact in the area where releases are planned.

The DSS suggested that *G*. cf. *brasiliensis* G1 is a safe biological control agent that can be released with minimal risk in Europe. Establishment of *G*. cf. *brasiliensis* G1 was considered very likely in a large part of the IAA because of the widespread distribution of its host, as well as climate conditions that are largely similar to its native range and other invaded areas in North America. However, a precise mapping of its establishment potential in the IAA could not be made because data on its climate requirements are lacking and the precise distribution of *G*. cf. *brasiliensis* G1 in Asia is still poorly known.

The negative environmental impact was considered minimal, mostly because the parasitoid is specific to Drosophila spp. infesting fresh fruits. In the laboratory, G. cf. brasiliensis G1 can occasionally attack closely-related species, such as D. melanogaster, a species only rarely found in fresh fruits (Girod et al. 2018b; Seehausen et al. 2020). In addition, during surveys in Asia, G. cf. brasiliensis were only obtained from Drosophila spp. in fresh ripe fruits that were collected directly from the plant and were never obtained from fruit baits (typically sliced banana pieces), indicating a high degree of habitat specificity under field conditions (Kasuya et al. 2013; Daane et al. 2016; Girod et al. 2018a; Giorgini et al. 2019). In Europe, the only Drosophila sp. occurring in fresh fruits is *D. suzukii*; therefore, field parasitism of other *Drosophila* spp. is likely to be very low and without negative impacts on their populations and their role in the ecosystem. The positive environmental impact is considered moderately important, mostly because of the expected reduction in pesticide applications, use of protective netting and mass trapping. Thus, it is clear that the positive environmental impacts largely outweigh the risk of causing negative environmental impacts on native biodiversity and ecosystem patterns and processes. This does not include the socio-economic impacts, which

would presumably be noteworthy if the parasitoid successfully controls *D. suzukii* in fruit production. Nevertheless, as in all classical biological control programmes, non-target effects should be carefully monitored through post-release studies.

Of particular interest was the fact that the DSS gave the possibility to consider undescribed cryptic species as potential BCAs, provided they can be unambiguously categorised. The fact that the different genetic groups (or cryptic species) of the *G*. cf. *brasiliensis* complex can be separated using molecular analyses and that only one genetic group of the parasitoid, *G*. cf. *brasiliensis* G1 is specific to *D*. *suzukii* larvae feeding in fresh fruits (Girod et al. 2018b; Seehausen et al. 2020) strongly supports justification for release of this biological control agent. Furthermore, considering that the introduction of the other genetic groups, *G*. cf. *brasiliensis* G3–G4, has a higher risk of non-target effects, because they also attack indigenous *Drosophila* spp. larvae in decomposing fruits in the region of potential introduction (e.g. North America or Europe) clearly demonstrates the value of DNA analysis to tease out genetic groups that cannot be separated through morphological characteristics (Nomano et al. 2017; Seehausen et al. 2020).

In conclusion, the DSS has demonstrated that *G*. cf. *brasiliensis* G1 is a safe biocontrol agent that can be released with minimal risk in Europe, while meaningful non-target risks were identified for other genetic groups of *G*. cf. *brasiliensis* (see Suppl. material 2).

Assessment of Cleruchoides noackae using the modified DSS

The Full assessment is presented in Suppl. material 3. The assessment was easy to follow. *Cleruchoides noackae* was used in other biological control programmes in South Africa, South America and Israel and was the object of several studies, which facilitated its assessment for release in Europe (Nadel and Noack 2012; FABI 2013; Barbosa et al. 2017). The specificity of *C. noackae* to the Thaumastocorinae subfamily, native and restricted to Australia (Lin et al. 2007; Cross 2009), minimises uncertainties related to its impacts on non-target species and native communities in Europe. The host specificity study from Portugal supports the conclusion of minimum uncertainty of impacts on non-targets. This study was included in the official application for releases, but the data are still unpublished. Although *C. noackae* was also assessed in other non-European countries (South Africa, Israel and Brazil) to our knowledge, the results have not yet been published.

An important point to note is that the three trophic levels involved in this system; the host plants, the insect pest and the parasitoid are all non-native to Europe and considering the specificity of the parasitoid, detrimental impacts of the releases on native species, native communities and conservation areas, are expected to be negligible. Based on this and on previous field and laboratory data, most questions regarding environmental risks were answered with minimal risk and low uncertainty. In this regard, the DSS provided strong arguments related to the minimal risk of releasing *C. noackae*. The questions for which answers were given with medium certainty were those on climate suitability and establishment probability. Data on the life cycle are

available from laboratory studies (e.g. Barbosa et al. (2018)), but there is not much field data, specifically on the ability of the parasitoid for overwintering in cold winters. This might limit its establishment or efficiency in the colder areas of southern Europe. Additionally, there are no studies on climate distribution models applied to this species. Still, its current distribution in the native and introduced range includes several regions climatically similar to southern Europe.

There are socio-economic arguments in support of biological control of T. peregrinus that were not considered in the assessment. Eucalyptus plantations are highly valued for the fast-growing nature of the trees and their production of foliage, essential oils, wood, biomass and raw material for the pulp industry. Currently, Eucalyptus plantations in Europe occupy an area of more than 1.8 million ha. Although the expansion of Eucalyptus plantation in the Mediterranean Region has sometimes been associated with negative environmental impacts (Tomé et al. 2021), Eucalyptus trees also have many positive environmental impacts related to soil conservation, climate amenity, carbon sequestration and provision of nectar and pollen for pollinators, amongst others (Branco et al. 2015; Tomé et al. 2021). In urban areas, these trees are often used in streets and gardens for amenity purposes. The control of T. peregrinus or of other pests affecting *Eucalyptus*, will thus have significant positive impacts. The alternative method of applying insecticides would be costlier and would imply negative environmental impacts. In contrast, no negative environmental impacts are expected with the release of *C. noackae*. Therefore, there is low uncertainty when considering the positive environmental impacts related to the reduction of *T. peregrinus*. It should be noted that the distribution of *Eucalyptus* plantations in Europe is mainly concentrated in Portugal and Spain, so overall, we consider the expected impact will be moderate.

The DSS demonstrated that *C. noackae* is a safe biological control agent that can be released with minimal risk in Europe and that the positive environmental impacts largely outweigh the risks or uncertainties surrounding its establishment. Still, the establishment should be carefully monitored through post-release studies.

Conclusions and recommendations

Both *G.* cf. *brasiliensis* G1 and *C. noackae* were successfully assessed with the modified version of the DSS for CBC of plant pests. No major issues were encountered during the assessments, most questions being answered with low levels of uncertainty. For both species, the outcome of the assessment was that the parasitoids were safe to release in the IAA, with the positive impacts exceeding the negative ones. The scheme would need to be further tested on agents that are less specific; however, there is evidence from unpublished assessments with polyphagous natural enemies where no major issues were apparent and the important risks were clearly highlighted.

The two parasitoids assessed in this study were well known due to previous releases in other regions in the case of *C. noackae* and because of extensive studies in Europe in the case of *G.* cf. *brasiliensis* G1. Potential CBC agents that are less studied would be more difficult to assess, resulting in higher uncertainties. The DSS could mention that species that have been well studied and are ready to be released are better suited to this assessment. On the other hand, the scheme could also be used at an earlier stage of a CBC programme to identify priorities for research, such as host range testing or climate matching models.

The goal of modifying the DSS was to build a version that is specifically and unambiguously made for assessing classical biological control agents of plant pests, but without changing the general aim and structure of the scheme and keeping other changes, for example, in the wording, to a minimum. However, the scheme could be further developed in various ways, outlined below.

1. Step 2. Assessment of probability of establishment could be further shortened. Several criteria for assessing establishment of the agent are still considered in our revised version in an effort to find a compromise, but most of them are more relevant for ABC or for CBC of weeds. In CBC of plant pests, the most relevant criteria are factor 1 about the presence of the hosts or prey and factor 3 about a suitable climate. Questions on other abiotic factors, on competition and natural enemies preventing establishment or on the adaptability of the BCA agent may be important in specific cases, but are often difficult to answer. Questions on management practices and control measures that may prevent establishment may be relevant, but all CBC practitioners will first release CBC agents for establishment at sites that are not managed with pesticides. The question on the presence or need of alternative hosts and other essential species is ambiguous and could be rephrased. For CBC, attacks of alternative hosts, i.e. those that occur at the same time as the target species, would be undesired, unless it is another pest that needs to be controlled. For some agents, the presence of an alternate host, i.e. a host that can be attacked before or after the occurrence of the target pest, may be crucial for the establishment in a new area and, thus, important for the success of a CBC programme. On the other hand, if the alternate host is native, it implies that non-target effects will occur. In most CBC programmes, no alternative nor alternate host is required for its success.

2. Step 3. Assessment of potential environmental consequences is based on a comparison and balancing between the negative (risks) and the positive (benefits) environment impacts. However, they are not assessed equally. In the positive impact assessment, only environmental criteria are included. In contrast, in the negative impact assessments, there are also four questions on negative impact on ecosystem services (3.02.12–3.02.15), based on the EFSA (2016) criteria. Impacts on ecosystem services implies socio-economic impacts. For example, provisioning ecosystem services includes provision of food, raw materials etc. In addition, impacts on cultural ecosystem services also clearly imply socio-economic impacts. Often, a decision to release a CBC agent takes into consideration the balance between environmental risks and socio-economic benefits. Thus, the questions of the DSS should explore both the negative and positive impacts for the environment, as well as from the socio-economic point of view. If possible, a positive socio-economic impact component should be included in

the DSS, for example, a set of questions determining the socio-economic benefits of BCA releases and the expected reduction in pest impact. If the scheme has to remain purely an environmental risk assessment, the questions on negative impacts on ecosystem services (3.02.12–3.02.15) should be removed, since the negative environmental impacts are sufficiently covered by other questions.

3. Finally, a well-defined rating system could be developed for possible negative and positive impacts by the CBC agent to facilitate the overall conclusion and make the final decision transparent and clear. In such a rating system, the level of uncertainties could be used to facilitate the overall conclusion derived from the DSS. For example, if uncertainty about potential non-target effects is high, the rating system could suggest declining the release application until the uncertainties are resolved. We acknowledge that this is a controversial subject, which has previously been tackled by using risks versus benefits matrices (e.g. Moeed et al. (2006); van Lenteren and Loomans (2006)). The main difficulty with such scoring systems is that the assignment of values is subjective and required total scores for decision-making may vary between jurisdictions.

Besides the use of the DSS by decision-makers, other proponents of a biological control agent release, researchers, for example, can use the document to identify and gather the information that needs to be provided in an application for releases to regulatory authorities. It is important to point out here that the DSS is not a substitute for a release application, but it could be used to develop a standard template for preparing an application for CBC of a plant pest.

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Supplementary material I

Decision-support scheme for release of classical biological control agents of plant pests – Environmental impact assessment (EIA)

Authors: M. Lukas Seehausen, Manuela Branco, Catarina Afonso, Marc Kenis Data type: docx

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Supplementary material 2

Assessment of Ganaspis cf. brasiliensis

Authors: M. Lukas Seehausen, Manuela Branco, Catarina Afonso, Marc Kenis Data type: docx

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Supplementary material 3

Assessment of Cleruchoides noackae

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Emerging tree diseases are accumulating rapidly in the native and non-native ranges of Holarctic trees

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Abstract

Emerging infectious diseases threaten natural and managed trees worldwide – causing reduced vigour, increased mortality and, occasionally, extirpation – yet we have little understanding of how emerging diseases have accumulated over time and how accumulation has varied by host species, host nativity and in different global regions. To address this challenge, I assembled over 900 new disease reports on 284 tree species in 88 countries and quantified how emerging infectious diseases have accumulated geographically and on different hosts. I show disease accumulation is increasing rapidly globally, with numerous recent years having nearly twice the number of new records as the twenty-year average and the number of new reports doubling every ~ 11 years. Of the tree genera assessed, *Pinus* had by far the most new diseases reported over the last several decades, likely reflecting both its large native range in the Northern Hemisphere and its wide use in forestry globally. Most hosts tend to accumulate more diseases in their native ranges than their non-native ranges, consistent with pathogen introductions causing most new diseases. Europe and Central Asia had the most accumulated emerging diseases, but accumulation is also increasing rapidly in East Asia. This work suggests that the impacts of emergent tree diseases are likely to continue to compound in the future and threaten native and planted forests worldwide.

Keywords

emerging infectious diseases, global trends, host jumps, non-native plants, plant pathogens, pathogen accumulation

Introduction

Emerging infectious diseases (EIDs) – defined as diseases that have, in the past several decades, expanded their host breadth, geographic range, increased in severity or are newly discovered, recognized or re-emerged (Anderson et al. 2004; Ghelardini et al.

2016) – are a threat to natural and managed plants. Particularly damaging EIDs over the past decades have threatened native plant populations with expiration (Anderson et al. 2004) and have reduced the yield and vigour of non-native plants cultivated for aesthetics, agriculture or forestry (Ristaino et al. 2021). Impacts on host populations have resulted in numerous EIDs reshaping forests around the globe. Perhaps the most well-known tree disease in North America, chestnut blight (caused by *Cryphonectria parasitica*) effectively eliminated chestnut as an overstorey tree in its native range in the Appalachian Mountains and facilitated a transition to oak/hickory dominated forests (McCormick and Platt 1980). Although most EIDs do not have such dramatic effects on their hosts, the continued emergence of new diseases increases the likelihood of particularly damaging diseases emerging, reducing host populations and shifting forest community composition. Ash dieback in Europe, Jarrah dieback in Australia and *Torreya* mycosis in Florida, for instance, are each causing similar host population declines that may result in host extirpation and co-extinction of associated species.

Increased global trade and connectivity and shifting environmental conditions have facilitated the emergence of many infectious diseases, with most EIDs being due to introductions and weather events (Anderson et al. 2004). EIDs due to introductions frequently cause small effects on hosts in their native ranges, likely due to long-term co-evolutionary history, but when exposed to novel hosts - often closely related to those in their native ranges - can cause mortality previously unseen in the native range (Loo 2009) as hosts may lack any evolved resistance to the pathogen (Ghelardini et al. 2017). Other pathogens can emerge from shifting biotic or climatic regimes, that may increase the likelihood of spread and establishment. Climate change can both create more favourable conditions for pathogens and stress host plants that, together, can facilitate attack by pathogens. As climates shift away from their historic norms, hosts and pathogens may need to shift their geographic ranges and the balance of their interactions may similarly shift (Corredor-Moreno and Saunders 2020). Warmer winters, greater precipitation and higher humidity may each act to, generally, improve conditions for pathogens, while high heat and drought may stress host plants making them less able to resist infection. Recent climate shifts, for instance, have likely increased the prevalence of Cronartium ribicola in portions of western United States and facilitated its spread to previously inhospitable areas (Dudney et al. 2021). Likewise, the increased frequency of climate-linked extreme events (Rahmstorf and Coumou 2011) also have the potential to increase the rate of EIDs (Rosenzweig et al. 2001), by creating unique conditions conducive to tree stress and favourable to pathogen populations. Numerous tree EID outbreaks have been linked to climate change or are predicted to be impacted by future climate change (e.g. Woods et al. 2005; Aguayo et al. 2014; Goberville et al. 2016; Kolb et al. 2016; Contreras-Cornejo et al. 2023), suggesting the possibility of even more damaging EIDs in the future.

Changing biotic communities have also facilitated the emergence of EIDs. Many pathogen and insect pest species have been known to track their hosts to new regions, which may then spill over to new hosts. Bonnamour et al. (2023), for instance, recently showed that non-native plant introductions have frequently preceded insect introduc-
tions, suggesting that non-native plant accumulation likely contributes in concert to pathogen spread and accumulation in new regions outside their historic ranges. Further, many pathogens are only known to affect their tree hosts outside their hosts' native ranges (Gougherty and Davies 2022b), which may then be transported back to the native range where it may damage native populations. Non-native trees offer a useful bellwether for identifying future pest threats before they have the opportunity to arrive in a host's native range. For instance, in a review of the fungal pathogens of European and Eurasian trees grown in Siberia, Tomoshevich et al. (2013) identified dozens of previously unknown fungal-host associations, a substantial portion of which were considered "high impact," that could threaten tree populations if transported back to tree's native ranges.

While much is known about the spread and emergence of some individual, particularly high impact, EIDs (e.g. Grünwald et al. 2012; Bulman et al. 2016; Burgess et al. 2017; Enderle et al. 2019; van der Nest et al. 2019; Futai 2021), less is known about the overall, general temporal trends of EID accumulation and how these trends vary by geographic regions, host species and host nativity. Understanding where and which hosts are most likely to accumulate pathogens can be important for future surveillance and management, especially as eradication often becomes unfeasible after pathogens begin to spread on the landscape (Smith et al. 2017). To address this challenge, I compiled hundreds of reports of EIDs on new tree hosts and in new geographic regions and compared the accumulation of EIDs in different global regions and in locations where hosts are native and non-native. I focused on a set of widely distributed tree genera, mostly native to the Holarctic, with large native and non-native ranges, that have been introduced (intentionally and not) to many different regions of the world. The "big data" approach used in this study helps to characterise the growing threat posed by EIDs and how this threat is unequally distributed regionally and by host species.

Methods

Literature search and data extraction

To find relevant reports of new EIDs, I searched the primary literature using multiple databases. Searches primarily involved identifying records where pathogens were identified on new hosts, new geographic regions or were reported to be increasing in severity. The plant pathology literature has a history of reporting such records as "First reports" which typically involve researchers describing the location, host and symptoms of the disease and the approach used to verify the pathogen. This often involves validating Koch's postulates (an established approach to demonstrate a causal link between a disease and suspected pathogenic microorganisms) for fungi, bacteria and nematodes, performing molecular techniques for viruses and phytoplasmas and morphological verification for parasitic plants. These new results are often published with titles such as "First report of [pathogen] causing [disease] on [host] in [locale]."

I used several approaches to find relevant reports of recent EIDs. First, I searched multiple online databases for "first report", "first record", "first occurrence", "newly reported", "for the first time" and "first finding". Searches were primarily conducted with PubAg and PubMed and Wiley Publishers for a select number of publication titles not included in PubAg. PubAg is a public catalogue of agriculture-related publications and was accessed by the PubAg API (https://pubag.nal.usda.gov/apidocs) with R statistical software (v.4.2.0). Searches involved querying article titles in seventeen plant-based journals including "Archiv für Phytopathologie und Pflanzenschutz", "Australasian plant disease notes", "Australasian plant pathology", "Bulletin OEPP", "Canadian Journal of plant pathology", "Crop protection", "European Journal of plant pathology", "Forest pathology", "Journal of general plant pathology", "Journal of plant pathology", "Microbial pathogenesis", "Phytoparasitica", "Phytopathologische Zeitschrift", "Plant disease", "Plant health progress", "Plant pathology" and "Plant Protection Science." Searches were limited to the years 2000 to 2022 to align with the temporal definition of emerging diseases and to capture the most recent EIDs. Although records from 2022 may be incomplete, as the final search was conducted in January 2023, these records were, nevertheless, included as they represent the most recently-confirmed EIDs. Any reports currently listed as a "First look", before publication in a journal issue, were assigned to 2022.

Next, I searched CAB Direct for the same terms as those above (i.e. "first report", "first record" etc.). CAB Direct is a unique resource as it indexes scientific publications, reports and conference abstracts (many of which, CABI states, are unavailable elsewhere) published in over a hundred countries and over 80 languages. Finally, I manually searched the table of contents of "Plant Disease" – Disease Notes, "New Disease Reports", "Forest Pathology" and "Plant Pathology" for relevant records. Although I attempted to be comprehensive in these searches by using multiple search terms and multiple unique databases, it is likely that some relevant EID reports were unintentionally omitted.

As this work was focused on tree species and, in particular, species that have extensive native and non-native ranges, I focused on a select number of species-rich host tree genera mostly native to the Holarctic, including *Abies, Acacia, Acer, Alnus, Betula, Carya, Castanea, Eucalyptus, Fagus, Fraxinus, Juglans, Larix, Picea, Pinus, Platanus, Populus, Pseudotsuga, Quercus, Robinia, Tectona, Thuja, Tilia, Tsuga* and *Ulmus.* Genera mostly from the Holarctic were selected because: (i) higher latitude species tend to have larger geographic ranges than low latitude species (i.e. Rapoport's rule) – thus, they many exist as natives or non-native in many regions, (ii) these genera represent some of the most widely grown species for forestry and cultivation and occasionally act as invasives and (iii) European (Lenzner et al. 2022) and North American colonialism have spread native trees of these regions to many other parts of the Globe, increasing the likelihood they may be exposed to new pathogens. Other genera (e.g. *Eucalyptus, Acacia*) were included because they tend to be widely planted for forestry.

After searching for all new disease reports, titles were searched for the common and scientific names of the tree genera listed above. Any record that matched was retained

for further analysis. Next, to partially automate the data gathering process, titles were searched for country names and pathogen names. I used the country-code package in R statistical software (v.4.2.0) (Arel-Bundock et al. 2018) for standardised country names and regional affiliations (based on World Bank Development Indicators regions). Pathogen names were downloaded from the CABI Crop Protection Compendium (CABI 2023), which maintains datasheets on > 10,000 plant pests with contemporary names. Records that did not have a matching country name or pathogen name were retained regardless and all records were checked manually to ensure the extracted host, pathogen and country names were accurate. Duplicate records from the same country were removed, with only the earliest record being retained. Thus, the retained records considered spread between countries, but not spread within countries unless, while spreading within a country, the pathogen became associated with a new host.

Host nativity assignment

Host nativity was determined using the GlobalTreeSearch (GTS; https://tools.bgci. org/global_tree_search.php), which maintains checklists of native trees for nearly all countries (Beech et al. 2017). For each country-host occurrence in the dataset, hosts were considered native in a focal country if: (i) the species was included in the GTS and (ii) the species was included in the checklist for the focal country. This two-step process was necessary as some hosts were not included in the GTS (e.g. because they were synonyms, hybrids or not distinguished below the genus-level). Any host species not included in the GTS were checked manually. In some instances, where hosts were only identified to the genus-level, they were considered non-native if no species of the genus occurred natively in the country (e.g. Eucalyptus hybrids outside Australasia). Otherwise, the nativity was left ambiguous and not included in any native/non-native comparisons. Hybrids followed a similar rule – if both parent species were known to be native to a country, the hybrid was considered native. If neither parent species was native, it was considered non-native and nativity was left ambiguous if one parent species was native and the other was not. In total, only a small percentage (3.8%) of hosts in the dataset were found to have ambiguous origins. All species names (both in the EID reports and GTS) were standardised to the Global Biodiversity Information Facility (GBIF) taxonomic backbone, using the rgbif package (Chamberlain and Boettiger 2017), before matching. The complete dataset is available in Suppl. material 2.

Analyses

I assessed the temporal accumulation of EIDs globally and separately for multiple geographic regions and host genera. Accumulation was calculated simply as the cumulative sum of new reports published since 2000. While I acknowledge publication may occur several years after sampling, which may itself be several years after the disease initially emerged, publication year was the only consistently reported time-stamp and represents the time the information became widely available. Exponential models

were fitted to accumulation curves to visualise increasing accumulation (concave upwards) versus saturation (concave downwards). An exponential model was chosen as the rate of new reports is expected to change over time, contingent on host species and geographic region and whether new reports are increasing or declining. Compared to a linear model, an exponential model should be able to capture the changing rate of accumulation over time. Models were fitted with the nls function in the stats package (R Core Team 2023), as $y \sim a \times \exp(b \times x) + c$, where y is the cumulative number of new reports and x is the number of years elapsed since 2000 (the first year of data collection). To facilitate interpretation and visualisation, in the below plots, x was back-transformed to year (by adding 2000). Model fit was estimated by pseudo R², calculated from the aomisc package (Onofri 2020). Approximate doubling time of new reports was calculated by fitting a logarithmic model: $log_2(new reports) \sim year +$ intercept and calculating the inverse of the slope estimate.

As most countries and tree genera have more native than non-native tree species, the accumulation curves and models were also plotted after standardising the cumulative sum by the number of native and non-native species. These plots and analyses thus represent the number of EID reports per species, thereby controlling for the unequal frequency of native and non-native species.

In addition to quantifying the temporal accumulation of EID reports, I also assessed the relationship between the number of EID reports and the accumulated number of total agricultural and biological documents published in literature, extracted from Scimago Journal & Country Rank (SCImago 2022) from 2000 to 2021. This analysis helped reveal how EID reports have increased with increased sampling and documentations, as represented by the number of citable scientific documents.

Results

In total, 962 host-pathogen-location EIDs were identified, from 2000 onwards, across 24 host genera (including > 280 species) and 88 countries. Globally, reports of EIDs have increased rapidly over time (Fig. 1) and in proportion with the expanding agricultural and biological literature (Suppl. material 1: fig. S1). New reports of EIDs on the focal genera, however, have not increased relative to the total number of new EID reports for all host species (i.e. including crops and other non-tree species) (Suppl. material 1: fig. S2). A median of 42 new EIDs were reported annually on trees of the 24 focal genera although 2019 had twice that number. The increase is seen both in regions where hosts are native and non-native although, to date, countries where species are native have accumulated more pathogens. Doubling time for new EID reports was approximately 11.2 years for all hosts, but 14.3 years for native hosts and 8.7 years for non-native hosts.

Interestingly, the patterns of accumulation differed by region (Fig. 2). Total accumulation was highest in Europe, both amongst native and non-native trees. This is likely due to a combination of increasing importation of non-native pathogens (Santini et al. 2013), well-developed international reporting systems (e.g. European Plant



Figure 1. a geographic distribution and **b** temporal accumulation of 962 first reports of tree EIDs since 2000 for 24 tree genera. Note the scale in (**a**) has been \log_{10} transformed and no reports of EIDs were found for countries coloured grey. Models in (**b**) were fitted as $y - a \times \exp(b \times x) + c$, where y is the cumulative number of new reports and x is the number of years elapsed since 2000 (the first year of data collection). To facilitate interpretation and visualisation, x was back-transformed to year (by adding 2000). Native and non-native reports in (b) do not always sum to the total, as numerous host species had ambiguous geographic origins.

Protection Organisation) and relatively small country sizes – where a pathogen may be considered 'new' even if it has been reported in an adjacent region (but different country). North America has similarly seen increasing pathogen accumulation, but growth is considerably slower in both native and non-native trees. Parameter estimates suggest accumulation is saturating in North America for non-native trees (Suppl. material 3). This slower accumulation could be due to relatively large country sizes (i.e. United States and Canada) – although many first reports for the US and Canada are at



Figure 2. Temporal accumulation of first reports of tree EIDs since 2000 for four geographic regions and 24 tree genera. Models were fitted as $y - a \times \exp(b \times x) + c$, where y is the cumulative number of new reports and x is the number of years elapsed since 2000 (the first year of data collection). To facilitate interpretation and visualisation, x was back-transformed to year (by adding 2000). See also Suppl. material 1: fig. S3.

the state/province level – but also effective pathogen/pest exclusion policies (e.g. Plant Quarantine Act of 1912; Plant Protection Act of 2000).

Pathogen accumulation on native and non-native trees in East Asia were similar to those in North America and Europe, but seemed to be increasing at a more rapid pace, perhaps due to increased introductions of non-native pathogens and improved reporting. Not surprisingly, accumulation of pathogens on natives in Latin America and Caribbean was slow and tended towards saturation (Suppl. material 3), as most of the genera investigated here were not native to this region. However, like East Asia, the accumulation on non-native plants is increasing rapidly, perhaps due to increased planting of non-native trees for forestry in this region. When standardised by the total number of native and non-native species, accumulation trends were similar in each global region, but the native and non-native curves for North America and East Asia and Pacific were nearly identical – indicating that EID accumulation per species, is similar for native and non-native trees in these regions (Suppl. material 1: fig. S3).

EID accumulation varied substantially amongst the genera assessed (Fig. 3), but like the regional patterns, accumulation was frequently higher in species' native, rather than non-native ranges (Fig. 4). *Pinus* provides a clear example of reports of new pathogens tending to be higher in the native region. Notably, however, when standardised by the total number of native and non-native species, the accumulation trends have



Figure 3. Frequency of first reports for 24 tree genera since 2000. Note the nativity classification corresponds to the nativity of particular host species, not the genus as a whole.



Figure 4. Temporal accumulation of first reports of tree EIDs since 2000 for four host genera with the greatest total number of EID reports in the dataset. Note the nativity classification corresponds to the nativity of particular host species, not the genus as a whole. Models were fitted as $y - a \times exp(b \times x) + c$, where y is the cumulative number of new reports and x is the number of years elapsed since 2000 (the first year of data collection). To facilitate interpretation and visualisation, x was back-transformed to year (by adding 2000). See also Suppl. material 1: fig. S4.

begun to converge in recent years (Suppl. material 1: fig. S4). *Eucalyptus* showed the opposite pattern, whereby pathogen accumulation is more rapid where it is planted outside its native range. *Eucalyptus* has a relatively narrow native range in Australasia, but has been planted in hundreds of countries abroad, exposing it to a broad range of new pathogens not present in Australasia (Burgess and Wingfield 2017).

Discussion

As has been found for non-native species accumulation (Seebens et al. 2017) and nonnative pests specifically (Aukema et al. 2010; Santini et al. 2013), there is little evidence of saturation in emergent tree disease accumulation globally. This is particularly worrisome for native plant populations as EIDs can lead to reduced growth, population reduction and, in severe cases, extirpation. Emergent disease on non-native plants present their own sorts of risk – not only could emergent disease on non-native plants spill over to native plants, as can happen when pathogens track their hosts to a new region, but hosts may then present a sort of biological pathway to the native range (Gougherty and Davies 2022b). For instance, the introduction of white pine blister rust into North America is believed to have been due to the importation of infected native white pines grown in Europe, where they are non-native (Hummer 2000). EIDs on non-native trees, thus, have the potential of being important bellwethers of future threats to trees in their native ranges.

The continued accumulation of EIDs implies diseases are likely to continue to spread and accumulate on new hosts - increasing the likelihood of severe outbreaks and host mortality. The increase in EID reports over the past several decades is likely due to a combination of increased global connectivity and increased reporting (Suppl. material 1: fig. S1). While national and international policies can and have been implemented to limit the spread of plant pathogens across borders (Santini et al. 2018) this only prevents one type of EID (i.e. those due to introduced pathogens). Even with complete exclusion of non-native pathogens, tree disease can still emerge from shifts in virulence or host range of native pathogens or changing environmental or climatic conditions increasing the opportunity or severity of host-pathogen interactions. Some recent work, for instance, suggests moisture and precipitation may contribute to recent pathogen outbreaks in plants (Romero et al. 2022) (but see Bebber 2022). Changes in climate could also shift the balance between host resistance and pathogen virulence (as predicted by the thermal mismatch hypothesis seen in wildlife species (Cohen et al. 2020)), leading to greater risk of disease outbreak amongst hosts and pathogens that have historically interacted. Understanding the link between environmental change and host-pathogen interactions may help identify the drivers of disease emergence which could improve monitoring and surveillance.

EID accumulation was not equally distributed amongst the genera assessed in this study. *Pinus* had the greatest number of accumulated EIDs – more than double that of any other genus assessed, except *Quercus*. The large number of EIDs on pines is likely

largely attributable to its large geographic range throughout the Northern Hemisphere and its use in forestry around the Globe. While *Quercus* has a similarly large distribution that spans much of the temperate region in the Northern Hemisphere, *Quercus* is also one of the most speciose plant genera (Global Tree Search lists 415 *Quercus* species globally (Beech et al. 2017)), which likely contributes to its large number of accumulated EIDs. The phylogenetic signal in many pests host ranges (Gougherty and Davies 2022b) suggests species-rich genera may be particularly sensitive to accumulating new diseases, as new pathogens may be able to easily jump amongst co-occurring congeners. *Eucalyptus* similarly has a large number of species (Global Tree Search: 723 species), but includes some of the most widely used trees in forestry and occurs in many countries as a non-native species. While it is unclear if hosts accumulate EIDs in similar ways to other pests (Dai et al. 2017), future work identifying the host traits associated with EID accumulation could help improve predictive pest association models and identifying hosts that may be most vulnerable to future EID threats.

Ash dieback

Many of the issues emblematic of EIDs are evident in the close examination of individual pathogens currently spreading on the landscape and encountering new hosts. The pathogen associated with ash dieback, for instance, one of the most frequently included in the dataset, highlights the impacts, challenges and future threats posed by EIDs. Over the past three decades, Hymenoscyphus fraxineus (anamorph: Chalara fraxinea) has spread rapidly throughout Europe, causing extensive mortality to its main host genus, Fraxinus. First reported in Poland in 1992 (Fig. 5) (Kowalski 2006), its ability to spread quickly from airborne spores (estimated at 30-70 km/yr) (Enderle et al. 2019) and human movement of infected plant material, the pathogen reached Spain in 2021 (Stroheker et al. 2021) and is now reported from at least 30 European countries. By the time the pathogen was scientifically described in 2006, it had already appeared and established in many European countries, challenging the implementation of potential eradication or management plans (Skovsgaard et al. 2017). Further, future shifts in the climate spaces used by the ash dieback pathogen and its European hosts introduce new uncertainty into where forests may be most impacted by the pathogen (Goberville et al. 2016). The ability of H. fraxineus to infect other, non-native trees particularly those native to North America, but planted abroad (Drenkhan and Hanso 2010) – highlights another dimension of its potential future impact. An introduction into North America could be particularly damaging as Fraxinus there are already experiencing extensive mortality from the emerald ash borer (Herms and McCullough 2014). While the ultimate effect of ash dieback on Fraxinus in Europe is uncertain, as some trees appear resistant to the pathogen (McKinney et al. 2014), when placed in a broader context, the epidemic is only one of the latest in a series that shows little sign of slowing. As EIDs continue to accumulate, over time, new high impact, unexpected pathogens are likely to emerge that will devastate host populations and reshape forests - a sequence of events that has repeated in forests around the globe.



Figure 5. Years of first detection of ash dieback in Europe. Dates were compiled from multiple sources (Timmermann et al. 2011; Keča et al. 2017; Milenković et al. 2017; Orton et al. 2018; CABI 2023).

Limitations

Despite searching thousands of records in the published literature, the estimates here of new disease emergences are certainly underestimated and this is likely for multiple reasons. First, recent work has consistently shown that pest documentations are strongly impacted by country wealth and scientific reporting (Gougherty and Davies 2022a). In line with this expectation, the accumulated number of EID reports increased linearly with the accumulated number of agricultural and biological documents in scientific literature (Suppl. material 1: fig. S1). While many of the host genera assessed here are native to relatively wealthy regions with well-developed reporting systems, nonnative ranges extend to all global regions, including less-wealthy countries that may not be well represented in the English-language scientific literature. This could partially explain why EID accumulation tended to be lower in regions where trees were nonnative. Furthermore, there is likely an impact-bias in the published records – that is, it is more likely that high-impact pathogens (i.e. those causing severe tree damage) will be investigated and ultimately included in a scientific report (Aukema et al. 2010). Host jumps or pathogen emergence in new geographic regions are likely to go unnoticed if

they cause minimal impact to their hosts. Likewise, many of the records assessed here were first observed in high-visibility locations, such as cities, universities and forest plantations. EIDs in wildlands not easily or frequently visited by the public or scientists are much more likely to go unnoticed for extended periods of time. While the true rate of global EID accumulation may never be known, these results suggest EIDs will continue to threaten natural and managed trees around the Globe in the coming decades.

Conclusion

Emerging infectious diseases pose a major threat to natural and planted trees around the Globe and are acting to reshape forests in the Anthropocene. EIDs are accumulating rapidly on Holarctic trees in both their native and non-native ranges, due to a combination of pathogen and tree introductions and environmental change. Although the rates of accumulation vary regionally and by host species, global trends show little sign of slowing, suggesting the impact of EIDs are likely to continue to compound and threaten tree populations globally.

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Supplementary material I

Supplementary images

Author: Andrew V. Gougherty

Data type: docx

- Explanation note: fig. S1. Relationship between the cumulative number of all citable scientific documents in the agricultural (ag.) and biological (biol.) literature and the cumulative number of EID reports. fig. S2. Proportion of all EID reports published in "Plant Disease" represented by Holarctic trees, used in this study. fig. S3. Temporal accumulation of first reports of tree EIDs since 2000 for four geographic regions and 24 tree genera. fig. S4. Temporal accumulation of first reports of tree EIDs since 2000 for four protect of tree EIDs since 2000 for four host genera with the greatest total number of EID reports in the dataset.
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Link: https://doi.org/10.3897/neobiota.87.103525.suppl1

Supplementary material 2

Publications of first reports of pathogens on 24 tree host genera published since 2000

Author: Andrew V. Gougherty

Data type: xlsx

Explanation note: Note the host species name may not match the host species name in the article if it did not match the GBIF taxonomic backbone.

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Link: https://doi.org/10.3897/neobiota.87.103525.suppl2

Supplementary material 3

Parameters estimates of an exponential model fit to the accumulation of new disease reports for various geographic, host, and nativity subsets

Author: Andrew V. Gougherty

Data type: xlsx

- Explanation note: Models were fit as $y \sim a \times exp(b \times x) + c$, where y is the cumulative number of new reports, and x is the number of years elapsed since 2000 (the first year of data collection).
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Assessing the invasion risk of traded alien ferns using species distribution models

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Abstract

Risk analysis plays a crucial role in regulating and managing alien and invasive species but can be timeconsuming and costly. Alternatively, combining invasion and impact history with species distribution models offers a cost-effective and time-efficient approach to assess invasion risk and identify species for which a comprehensive risk analysis should take precedence. We conducted such an assessment for six traded alien fern species, determining their invasion risk in countries where they are traded. Four of the species (Dicksonia antarctica, Dryopteris erythrosora, Lygodium japonicum, and Phlebodium aureum) showed limited global distributions, while Adiantum raddianum and Sphaeropteris cooperi had broader distributions. A. raddianum, however, was the only species found to pose a high invasion risk in two known trade countries - the USA and Australia - and requires a complete risk analysis to determine the appropriate regulatory responses. Dicksonia antarctica, Phlebodium aureum (for New Zealand), and Dryopteris erythrosora (for the USA) posed a medium risk of invasion due to the lack of evidence of impacts, and a complete risk analysis is thus deemed less crucial for these species in these countries. For other species, suitable environments were not predicted in the countries where they are traded, thus the risk of invasion is low, and a complete risk analysis is not required. For species in countries where suitable environments are predicted but no trade information or presence data are available, risk assessments are recommended to better determine the risk posed. Despite the relatively limited potential global distribution of the studied ferns relative to other major plant invaders (e.g., Pinus spp. and Acacia spp.), their history of invasion, documented impacts in pristine environments, and high propagule pressure from trade warrants concern, possibly necessitating legislative and regulatory measures in environmentally suitable regions.

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Keywords

early detection, environmental suitability, horticultural trade, invasion risk, MaxEnt, risk analysis

Introduction

The intentional or unintentional dispersal of species into areas outside of their native range is facilitated by various pathways of introduction which may be natural or human-mediated (Wilson et al. 2009). In particular, horticultural trade has been shown to be the primary source of introductions of invasive alien plants across the globe (Dehnen-Schmutz et al. 2007a; Dehnen-Schmutz and Touza 2008; Pyšek et al. 2011; Seebens et al. 2015; van Kleunen et al. 2018). Terrestrial true ferns (Polypodiophyta; hereafter 'ferns') have been a popular group in horticultural trade for centuries (Birkenhead 1897; Allen 1969; Whittingham 2010) and remain popular in trade today (de Winter and Amoroso 2003). Furthermore, various aspects of horticultural trade, such as a high market presence and trade via e-commerce, directly influence the invasion success of traded alien ferns (McCulloch-Jones et al. 2021). Many horticulturally popular ferns are successfully invading and negatively impacting various parts of the globe, for example, Sphaeropteris cooperi which has extensively invaded Hawaii and displaced native populations of the tree fern Cibotium glaucum (Chau et al. 2013); and Lygodium *japonicum* which is classified as a species of European Union concern (EU Commission 2022) and identified as an A1/A2 pest, requiring regulation and quarantine, by the European and Mediterranean Plant Protection Organization (OEPP/EPPO 2022).

The most cost-effective means of curbing invasions is through preventative action prior to introduction (IUCN 2000; Timmins and Braithwaite 2003; Hulme 2012; Larson et al. 2020; Martinez et al. 2020) or through early detection systems promoting rapid action post-introduction or following escape from confinement (Matthews et al. 2017; Reaser et al. 2020). Risk analysis is a comprehensive process used to determine the level of risk posed by invasive alien species and directly informs the appropriate management and regulatory response. In many countries these analyses are legally required for regulatory decisions regarding alien species, for example, in South Africa, the outcomes of risk analysis are used to determine whether a species is regulated and how (e.g., through trade restrictions or permitting) (Kumschick et al. 2020). Risk analysis is a systematic and robust evaluation that comprises various components, which can be grouped into four stages: hazard identification, risk assessment, risk management, and risk communication (Kumschick et al. 2020). However, as risk analysis is expensive and labour intensive, identifying species that should undergo this process is essential to best direct scare resources. Risk assessments provide a time and cost-effective means to aid in the detection and anticipation of potentially problematic species that require attention and for which a complete risk analysis may be needed to inform regulation and legislation (Chai et al. 2016; Carboneras et al. 2018, Kumschick et al. 2020).

Risk assessments comprise the initial steps of risk analysis and generally consider the likelihood of invasion alongside consequence (negative environmental or socio-economic

impacts) (Kumschick et al. 2020). Although all risk assessments generally cover these aspects, they may comprise different components depending on the approach adopted (sensu Kumschick et al. 2020). Risk assessment approaches include: trait scoring (species traits are used to predict potential for harm), statistical approaches (a trait approach combined with statistical or machine learning to determine likelihood of invasion), decision trees (a tracking system of questions and answers designed to lead to a decision), rapid screening (a quick assessment performed on a large number of species for which limited information is available), detailed approaches (a targeted approach which most closely resembles a risk analysis, requiring a substantial amount of data, management considerations, and stakeholder perceptions), or mechanistic approaches (completed for smaller groups of species evaluating the likelihood of species surpassing invasion barriers, and determining their potential impact). Recent studies applying a mechanistic approach have used similar sets of criteria to assess the risk posed by alien species, namely, a history of invasion elsewhere, invasion status (i.e., introduced and not yet naturalised, naturalised, or invasive) in the region of interest, evidence of environmental or socioeconomic impact in the invaded range, and climate or environmental suitability of the region of interest (Carboneras et al. 2018; Bayón and Vilà 2019; Reaser et al. 2020).

The consideration of the climatic or environmental suitability of the receiving region for the taxon in question can greatly enhance the predictive capacity of risk assessments (Beaumont et al. 2014; Chai et al. 2016; Matthews et al. 2017; Roy et al. 2018), and can be used to inform both preventative and reactive management responses. For example, species distribution models or climate matching techniques can detect habitats or regions that are suitable for a species, but where it does not yet occur or has not yet invaded – where preventative measures can be put in place – but can also help direct detection efforts to identify previously undetected populations in suitable habitats where reactive measures may be necessary.

Species distribution modelling (SDM) is increasingly used to predict the potential distributions of alien species and identify sites that are climatically or environmentally suitable for them. These models have been extensively applied across various taxa from marine life to insects and terrestrial plants, and at various scales from local to global (Robinson et al. 2011; Kumar et al. 2015; Mainali et al. 2015; Santamarina et al. 2019; Lamelas-López et al. 2020; Venter et al. 2021). For alien ferns, SDMs have been adopted in only a small number of studies, most of which are at a fine spatial scale (i.e., at a country level or for regions within countries) (Goolsby 2004; Volin et al. 2004; Bystriakova et al. 2014; Akomolafe and Rahmad 2019). These studies have shown that alien ferns can expand their invaded ranges under present-day environmental conditions. For example, the distribution of an emergent invasive fern, *Cyclosorus afer* (H. Christ) Ching, is predicted to expand to an area of more than 25 000 km² across four states in Nigeria (Akomolafe and Rahmad 2018), and in Florida, the invasive fern, *Lygodium microphyllum* (Cav.) R.Br., could become widely established throughout the Everglades (Goolsby 2004; Volin et al. 2004).

Alien ferns are generally understudied and thus are poorly represented in official plant species inventories and in invasive alien plant regulatory lists. Therefore, to detect alien fern species that have been introduced through trade, McCulloch-Jones et al. (2023) scanned horticultural catalogues for selected trading countries (Canada, the United States of America, the United Kingdom and the Republic of Ireland, South Africa, Australia, and New Zealand). They identified a total of 382 traded fern species and determined their invasion status in the countries in which they are traded, and success or failure of invasion elsewhere. This resulted in a list of 35 alien fern species that have a history of invasion elsewhere and that are introduced but not yet naturalised in the country in which they are traded. It was concluded that these species require SDMs to better assess their invasion risk and to determine the need for a complete risk analysis in countries where they are traded.

We selected six of these alien fern species and used SDMs to determine their potential global distribution. We subsequently considered the results of these models alongside information on the species' i) invasion status in the countries in which they are traded, ii) invasion history elsewhere, and iii) environmental or socio-economic impacts in their invaded range, to categorise each species, per trading country, in terms of the level of risk posed, and suggest the necessary response in terms of the need for risk analysis. We also indicate additional countries across the globe where the species are not yet known to occur and where risk assessment is necessary.

Methods

Study species

The six study species all have a history of invasion in numerous countries, are traded in several of the study countries (Canada, the United States of America, the United Kingdom and the Republic of Ireland, South Africa, Australia, and New Zealand), and have been introduced but are not yet naturalised or invasive in the countries where they are traded (McCulloch-Jones et al. 2023). The selected study species are further not associated with major taxonomic complexities and are not often mis-identified, issues that could lead to highly unreliable distribution data. For example, the fern *Polypodium vulgare* L. is fraught with considerable taxonomic issues (Haufler et al. 1995) and consequently, the current distribution data for this species in the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/) indicates an extensive occurrence in North America, yet the species *sensu stricto* is not known to occur there (in lit. Christopher Haufler, 03 July 2022). Species identification errors may lead to under-or-over predictions in models and may shift predictions to favour the environmental suitability of the 'contaminating species'. This can further influence the practical applicability of the SDM (i.e., misinformed management suggestions) (Costa et al. 2015).

Modelling procedure

Maximum entropy modelling (MaxEnt) was used for the SDMs in this study as various analyses have proven MaxEnt to be a reliable predictive approach that often

outperforms other methods in terms of the accuracy of the predictions, particularly for those related to biological invasions (Elith et al. 2006; Merow et al. 2013; Mainali et al. 2015). Furthermore, MaxEnt is useful in that it uses presence data as well as formulated background data as a substitute for true absences – which are difficult to obtain due to the lack of systematic survey data – to forecast the distribution of species (Zaniewski et al. 2002; Phillips et al. 2004, 2006, 2009; Phillips and Dudík 2008; Merow et al. 2013; Fourcade et al. 2014). Below we have described the modelling procedure, but a more comprehensive account is provided in Suppl. material 1(1).

Collection and preparation of species occurrence records

When developing SDMs for alien species it is recommended to include occurrence records from both the native and introduced ranges (Jiménez-Valverde et al. 2011; Mainali et al. 2015; Barbet-Massin et al. 2018). Alien species are often not in equilibrium with their environment in their introduced range (and, in some cases, in their native range). Therefore, models that consider only the introduced range (or, in some cases, only the native range) can produce predictions that do not adequately estimate the potential introduced range of the species (Fernández and Hamilton 2015; Srivastava et al. 2019), and are thus less useful in the context of biological invasions. Accordingly, species occurrence records from the native and introduced ranges were used in this study to build the SDMs. Data for all species were obtained from the GBIF (see Suppl. material 1(2) for details). Searches for occurrence records were performed using the currently accepted species name (see Table 2) according to the GBIF taxonomic backbone (https://doi.org/10.15468/39omei). The searches yielded occurrence records listed under the currently accepted name of each species, as well as those listed under commonly applied synonyms. For example, the full dataset for Sphaeropteris cooperi (F.Muell.) R.M.Tryon contained results for synonyms including Cyathea cooperi (F.Muell.) Domin, Cyathea brownii var. cooperi (Hook. ex F.Muell.) Domin, and Alsophila cooperi F.Muell. The total number of occurrence records obtained per species ranged from 3 237 records to 18 996 records (Suppl. material 1(2)).

The quality of the occurrence records for each species was assessed using the packages Biogeo (Robertson et al. 2016) and CoordinateCleaner (Zizka et al. 2019) in R version 4.1.0 (R Core Team 2020). Records that were errors (i.e., records that fall into the sea), environmental outliers (i.e., records that were far away from the rest of the records in environmental space, such as records of ephemeral populations, individuals in cultivation, or where there are errors in the co-ordinates, see Robertson et al. 2016), and centroids (i.e., records at the centre of countries), as well as those that were missing co-ordinates were identified and removed. Duplicate records were removed to avoid pseudo-replication, as were records that were too imprecise for the analysis at 2.5 arc minutes (-4.5 km² at the equator). We subsequently mapped the occurrence records for each species to identify instances where occurrence records were severely clumped and where thinning would be required to account for sampling bias, however, none were identified. We further compared the maps of the downloaded occurrence records with currently documented information regarding the range of each species to ensure that the full known range of each species was accounted for in the models, and to ensure that erroneous records were not retained during the cleaning process (for more details see Suppl. material 1(1)). The total number of occurrence records in the cleaned datasets for each species ranged from 886 to 3 356 records (Suppl. material 1(2)), which provided enough records for modelling (SDM predictions are generally regarded as not consistent if built using fewer than 30 occurrence records; Wisz et al. 2008).

Selection and preparation of predictor variables

Although ferns are considered habitat specialists, as a group, they are known to have similar broad environmental preferences and generally select for wet habitats with moderate temperatures (i.e., avoiding temperature extremes) (Kessler 2010). Therefore, we used a standard set of predictors to model the distributions of all the selected fern species. The selection of candidate predictor variables was informed using a multi-pronged approach whereby we, i) considered the ecological requirements and limitations of ferns in general, namely, light exposure, temperature extremes and water availability (Kessler 2010); ii) considered the predictor variables that were highlighted as being important in studies that have performed species distribution modelling for ferns at global (Christenhusz and Toivonen 2008; Brummitt et al. 2016) or finer scales (Lehmann et al. 2002; Bystriakova et al. 2014; Akomolafe and Rahmad 2019); and iii) noted if the predictor variable is also considered as 'state-of-the-art' (SOA) - those that are commonly used in SDMs for plant species (Petitpierre et al. 2017). When the environmental niches of multiple species are to be modelled with a standard set of predictors, it is suggested that SOA variables are used to build SDMs to ensure transferability and reduce computing requirements (Petitpierre et al. 2017). Nine candidate predictor variables were ultimately selected (see Table 1 for further justification for the selection of these variables) to predict the global distribution of the six study species. The candidate predictors included one landscape variable, land cover, which was acquired from the ESA CCI Land Cover project (http://maps.elie.ucl.ac.be/CCI/viewer/download.php) (Suppl. material 1(3)), and eight bioclimatic variables, which were acquired from the WorldClim dataset (WorldClim 2.1; Fick and Hijmans 2017). Befittingly, seven of the candidate bioclimatic predictors were also SOA variables (Table 1). All variables were downloaded at or converted to a 2.5 arc-minute spatial resolution for the purpose of modelling.

Co-linearity can be detrimental to the accuracy of SDM predictions (Dormann et al. 2013; Petitpierre et al. 2017). Accordingly, for each species we used the Pearson correlation coefficient ('cor' function in R) to test for correlation amongst the candidate variables. In cases where variables were co-linear (|>0.75|, Dormann et al. 2008) we opted to retain variables that represented extremes. As a precautionary addition, we used univariate generalised additive models (GAMs) (see West et al. 2016), to determine the percentage deviance explained by each predictor, and ensured that all statistically significant variables were not excluded. GAMs were performed using the package *MuMin* (Barton 2023). Consequently, of the nine candidate predictor variables, seven

Variable	Description	Data type	Motivation for selection with regards to ferns		
Landscape variable					
Land Cover	Land cover map including 30 classes describing habitat and percentage canopy cover, e.g., tree cover, broadleaved, deciduous, closed (> 40 %); or tree cover, needle-leaved, evergreen, open (15–40 %) (full details, Suppl. material 1)	Categorical	Accounts for the habitat and light requirements of ferns. Although many species of fern can withstand full sun and occur in bare areas, ferns are most commonly associated with shaded habitats, often in woodlands and forests (Kessler 2010).		
WorldClim	n bioclimatic variables				
Bio 1	Annual Mean Temperature (°C)	Continuous	An important predictor of fern richness and phylogenetic diversity at continental scales (Nagalingum et al. 2015). State-of the-art-variable (SOA; Petitpierre et al. 2017) commonly used to predict the distribution of plant species.		
Bio 4	Temperature Seasonality	Continuous	As for annual mean temperature. SOA.		
Bio 10	Mean Temperature of the Warmest Quarter (°C)	Continuous	Representative of climatic extremes. Furthermore, although the global distribution of ferns is generally associated with warmer areas in the tropics (de Winter and Amoroso 2003) only a few species are adapted to extreme hot temperatures (Hevly 1963). SOA.		
Bio 11	Mean Temperature of the Coldest Quarter (°C)	Continuous	Representative of climatic extremes. A limited number of fern species are adapted to survive sub-zero temperatures, and the majority of species do not show frond freezing tolerance (Fernández-Marín et al. 2021). SOA.		
Bio 12	Annual precipitation (mm)	Continuous	The variable has been identified as one of the most important predictors of fern richness and phylogenetic diversity at continental scales (Nagalingum et al. 2015). Additionally, water is essential for sexual reproduction in ferns (Sharpe and Mehltreter 2010). SOA.		
Bio 15	Precipitation seasonality (mm)	Continuous	As for annual precipitation. Additionally, fern occurrence and diversity are strongly associated with areas with many days of rain per year (Kessler 2010). SOA.		
Bio 16	Precipitation of the Wettest Quarter (mm)	Continuous	As for precipitation seasonality. Representative of climatic extremes. SOA.		
Bio 17	Precipitation of the Driest Quarter (mm)	Continuous	Representative of climatic extremes. The different life stages of ferns show varying levels of desiccation tolerance (López- Pozo et al. 2018), with approximately only 5–10 % of all fern species in the sporophyte form exhibiting desiccation tolerance (Hietz 2010).		

Table 1. Candidate predictor variables selected for modelling the potential distribution of the six alien fern species considered in this study.

(land cover, temperature seasonality, mean temperature of the warmest quarter, mean temperature of the coldest quarter, precipitation seasonality, precipitation of the wettest quarter, and precipitation of the driest quarter) were used to build SDMs for each of the six study species.

Background data generation

It has been recommended that the sites from which background records are selected should be unsuitable for the species, but should be near to the limit of what is suitable

(Jiménez-Valverde et al. 2011), and should be an intermediate distance from presence records (VanDerWal et al. 2009). Selecting background records in this way is more likely to result in SDMs that can correctly classify suitable from unsuitable sites (Jiménez-Valverde et al. 2011). Accordingly, background points for each species were selected from a restricted area that was, i) environmentally similar to sites where the species occurs, as identified using a map of the Köppen-Geiger climate zones (Kottek et al. 2006); and ii) within 500 km of the occurrence records based on the potential long-range wind and water dispersal of fern spores (Peck et al. 1990), but excluding areas within 5 km of the occurrence records as we assumed that while the organism has not been recorded at these sites, it likely occurs there (the majority of fern recruitment occurs within 2 m of the parent plants; Rose and Dassler 2017). Using a large number of randomly selected background records (e.g., 10 000 records) has been shown to greatly improve the predictive accuracy of MaxEnt models (Phillips and Dudík 2008; Barbet-Massin et al. 2012, 2018). Accordingly, we randomly selected 10 000 background points from the selected areas for each species, with one point per grid cell (i.e., no duplicates).

MaxEnt models

All models were built using MaxEnt Version 3.4.4 (http://biodiversityinformatics. amnh.org/open source/MaxEnt/; Phillips et al. 2004). In MaxEnt the choice of feature types and setting of the regularisation parameter (RM) are important considerations in the development of predictive models that involve transferal (i.e., when the potential distribution is estimated in a different time period or region than that where the data are from) (Phillips and Dudík 2008). Therefore, we modelled the distribution of the species using various features (e.g., hinge features only, and auto-features), and regularisation parameter settings, and monitored the impact on the predictions and model performance. The final, presented models were run using all features (auto-features), with the RM set to 1.5, which produced smoother, more ecologically plausible response curves (i.e., based on the general shapes of species-environment relationships - a typically smooth bell-shaped curve that does not have several minima or maxima; Austin 2007; Merow et al. 2013; Hannemann et al. 2016). SDMs produced using smoothed fitted functions have outperformed more complex models when modelling the distributions of alien species (Elith et al. 2010). Clamping was also used to avoid extrapolations of the SDM into novel environments.

All models were run using five-fold cross validation (as all records are used for training and testing in this method) and model performance was evaluated using multiple methods, i) evaluating the Area Under the Curve (AUC) statistic, ii) calculating the Continuous Boyce Index (CBI), iii) assessing the fitted response curves; iv) considering the sensibility of the model in terms of fern ecology (Austin 2007); and v) scrutinising the multivariate environmental similarity surface maps (MESS maps). The relative contribution of each environmental predictor in the model for each species was gauged using the Jack-knife test in MaxEnt which provides the percent contribution

of each variable to the model (Phillips et al. 2006). The potential global distribution of each species was mapped using QGIS 3.16.9, with the cleaned occurrence records used to produce the models for each species overlayed onto the prediction.

Risk assessment

Information on the invasion status of the study species per trade country and their invasion history (elsewhere) was taken from McCulloch-Jones et al. (2023) who largely obtained information from the global database of alien ferns (Jones et al. 2019) with updated data from published literature, herbarium records, and alien and invasive plant lists (e.g., the Global Register of Introduced and Invasive species; https://griis. org/). All study species have the status of introduced (i.e., present, but not yet naturalised, or invasive) in the trade countries, but their presence outside of captivity or cultivation is uncertain. To account for the history of invasion, species' invaded ranges were considered, while information on recorded impacts was obtained by searching the published and grey literature for evidence of environmental or socio-economic impacts of the study species in their invaded ranges (Table 2). We then closely scrutinised the SDM maps for instances where, i) suitable environments were available for the species in the trading countries (i.e., Canada, the United States of America, the United Kingdom and the Republic of Ireland, South Africa, Australia, and New Zealand), ii) where predictions indicated the possibility for expansion of the current range, and iii) where suitable habitat was predicted in regions outside of a species' current known range of occurrence. This information on invasion history, impact, and potential distribution was used to categorise species as per the level of risk posed, with each category aligned with a suggestion in terms of the requirement for risk analysis, namely, high risk- complete risk analysis needed; medium risk- risk analysis may be necessary, but not crucial, resources are better focussed on high risk species; and low risk- no further analysis necessary (see Table 3, as well as the decision tree presented in Suppl. material 1(4)). Lastly, we identified countries (or regions) other than the trading countries where suitable environments occur, but where the study species are not yet known to be present. Further work (research and subsequent risk assessment) is required in these regions in order to determine the risk posed, and whether risk analysis is required.

Results

Model performance

For all the models AUC values ranged between 0.76 and 0.95, indicating moderate to high performance, and the CBI values were > 0.97 indicating that predictions were consistent with the distribution of the occurrence records (Table 4). We noted few instances where occurrence records were found in areas that were predicted to have

Table 2. The six invasive alien fern species selected for the study, their native and invaded ranges, descriptions of the climates and habitats in which they occur, trading countries for which species distribution modelling is required according to McCulloch-Jones et al. (2023), and evidence obtained from the literature regarding the species' environmental or socio-economic impacts anywhere in their invaded ranges. CA– Canada, USA– United States of America, UK & RI– the United Kingdom and the Republic of Ireland (these were assessed jointly), ZA– South Africa, AU– Australia (AU), and NZ– New Zealand.

Species	Native range	Invaded range	Climate	Habitat	Trading countries in which species distribution modelling is required	Impact in invaded range
Adiantum raddianum C.Presl	Mexico to South America	Invaded Hawaii (Wilson 1996; DeMattos 2021), South Africa (Crouch et al. 2011; Jones et al. 2020), and parts of Europe (Keil et al. 2010)	Tropical and temperate	Herbaceous, terrestrial, lithophytic	CA, USA, AU	Displaces native species
Dicksonia antarctica Labill.	South-eastern Australia	Naturalised in the United Kingdom (Clement and Foster 1996) and Sri Lanka (Ranil et al. 2014), and has invaded São Miguel Island, Portugal (Arosa et al. 2012)	Temperate	Tree fern, terrestrial	CA, USA, NZ	None reported
Dryopteris erythrosora (D.C.Eaton) Kuntze	Eastern Asia	Naturalised in France and Belgium (Randall 2017) and is slowly but progressively invading various states of the USA (Rothfels et al. 2012; Umstead 2018; Wyatt 2020)	Temperate	Herbaceous, terrestrial	CA, USA, UK & RI, AU, NZ	None reported
Lygodium japonicum (Thunb.) Sw.	Asia	Invasive in south-eastern USA (Schmitz et al. 1997), Australia (Randall 2017), and South Africa (Jones et al. 2020)	Tropical and sub- tropical	Herbaceous, climbing	CA	Impacting the economic benefits of pine plantations and smothers indigenous vegetation
Phlebodium aureum (L.) J.Sm.	South- eastern USA, Caribbean, South America	Invasive in Australia, South Africa (Crouch et al. 2011; Jones et al. 2020), and Hawaii (Wilson 1996)	Tropical and sub- tropical	Herbaceous, epiphytic, terrestrial	NZ	None reported
Sphaeropteris cooperi (F.Muell.) R.M.Tryon	Eastern Australia	Invasive in Hawaii (Medeiros et al. 1992), South Africa (Jones et al 2020), and New Zealand (Heenan et al. 2004)	Temperate, tropical, and sub- tropical	Tree fern, terrestrial	US, U <u>K & RI</u>	Displaces native species and changes soil and plant nutrient dynamics

Table 3. Categorisation of alien plant species in the horticultural trade in terms of the risk posed. The categorisation is based on three primary criteria: history of invasion, evidence of impact; and environmental suitability. Each level of risk is aligned with a suggestion in terms of the requirement for risk analysis. See also Suppl. material 1(4) for a decision tree tracking this process.

Level of risk posed	Criteria	Requirement for risk analysis
High	Invasive or potentially invasive (i.e., invasive somewhere in the world) species for which suitable environments are available in the focus country, and for which impacts are known in its invaded range.	Complete risk analysis needed
Medium	Invasive or potentially invasive (i.e., invasive somewhere in the world) species for which suitable environments are available in the focus country, but for which no impacts have been recorded in its invaded range.	Risk analysis may be necessary, but resources are better focussed on high risk species
Low	Invasive or potentially invasive (i.e., invasive somewhere in the world) species, but for which no suitable environments exist in the focus country.	No further analysis necessary

Table 4. Model evaluation statistics for the SDMs for six selected fern species in trade. Results for both the Area Under the Curve (AUC) and Continuous Boyce Index (CBI) are shown.

Species	AUC	CBI
Adiantum raddianum	0.76	1
Dicksonia antarctica	0.88	1
Dryopteris erythrosora	0.88	0.97
Lygodium japonicum	0.85	1
Phlebodium aureum	0.91	1
Sphaeropteris cooperi	0.95	1

low suitability (*Dryopteris erythrosora* and *Sphaeropteris cooperi* in eastern USA, and *Dicksonia antarctica* along the British Isles and eastern USA). The MESS maps for each species indicated that the models did not extrapolate into novel environments. The vast majority of the response curves were plausible based on expectations of the general shapes of species-environment relationships (Suppl. material 1(5)). The response curves for two of the predictors, namely, temperature seasonality and precipitation seasonality did, however, appear slightly truncated or irregular in some of the models.

Variable contribution

The Jack-knife test of variable contribution showed that the most important predictor differed among the species, but land cover was the most important predictor for more than one species (*D. antarctica* and *P. aureum*) (Table 5). Mean temperature of the coldest quarter proved important for *A. raddianum*, precipitation of the wettest quarter for *D. erythrosora*, precipitation of the driest quarter for *L. japonicum*, and temperature seasonality for *S. cooperi*. Precipitation seasonality was the least important predictor for all species. On average, precipitation of the driest quarter and land cover contributed the most to the models.

	Land Cover	Temperature seasonality	Mean temperature of the warmest	Mean temperature of the coldest	Precipitation seasonality	Precipitation of the wettest quarter	Precipitation of the driest quarter
Adiantum raddianum	4.3	8.7	8	39.7	1	7.5	30.8
Dicksonia antarctica	67.4	14.5	12.7	0.2	0	0.2	5
Dryopteris erythrosora	9.4	7.1	18.4	3.7	0.1	39	22.3
Lygodium japonicum	9.4	15.1	0.6	1.3	0.2	17.9	55.5
Phlebodium aureum	35	0.3	0.1	28.7	10.6	1.6	23.7
Sphaeropteris cooperi	17	25.1	12.6	21	0.1	9.3	14.9
Average contribution	23.8	11.8	8.7	15.8	2	11.7	25.3

Table 5. Average percent contribution of the environmental predictors used in the SDMs for each species. The most important predictor for each species is in bold.

Potential species distributions

The predicted potential global distributions for most species spanned relatively few continents, with the exception of A. raddianum and S. cooperi for which suitable environments were predicted over several continents and in a greater number of countries across the globe when compared to the other study species (Fig. 1). For these two species, relatively large areas of suitable environment were predicted in many countries outside of their current introduced ranges, including southern Brazil, central African countries, and Madagascar for S. cooperi, and the south-eastern coast of Australia, countries in east Africa and southern Asia, the Western United Kingdom and Ireland, and Madagascar for A. raddianum. For L. japonicum, relatively large areas of suitable environment were predicted in a few countries where the species does not occur, namely southern Brazil, Uruguay, and Paraguay. Based on our models, it appears that for the remaining species predicted environments beyond the current introduced ranges have a relatively low suitability and aren't particularly expansive (Fig. 1). These include Slovenia, Croatia, and Montenegro for D. erythrosora; southern Brazil and Taiwan for D. antarctica; and east Africa and southern China for *P. aureum*. In terms of potential range expansion in regions where the species already occur, suitable, unoccupied environments are available for A. raddianum in east-central Africa and along the coasts of Spain, Portugal, France, Australia, New Zealand, and South Africa; large parts of the north-eastern coast of South Africa for S. cooperi, and south-eastern USA for L. japonicum.

Requirement for risk analysis

Our literature search showed that environmental or socio-economic impacts have been recorded for three of the study species (*A. raddianum*, *L. japonicum* and *S. cooperi*;



Figure 1. The global potential distribution of six invasive alien fern species as predicted by the species distribution models. In some instances, the identified suitable environments are not easily observed or have been superimposed with multiple species occurrence records. Insets have been used in these cases to improve visibility.



Figure I. Continued.

Table 6. Requirement for risk analysis of species in the countries in which they are traded based on, i) their invasion history, ii) invasion status in the country (all introduced and not yet naturalised or invasive), iii) whether the species is known to have impacts in its alien range, and iv) the availability of suitable environments according to the species distribution models (see Table 1). Trading countries are CA – Canada (CA), USA – the United States of America, UK & RI – the United Kingdom and the Republic of Ireland (considered jointly), ZA – South Africa, AUS – Australia, and NZ – New Zealand.

Species	Country	Risk rating and requirement for risk analysis		
Adiantum raddianum	CA	Low risk – no need for a risk analysis		
	USA	High risk – needs a complete risk analysis		
	AUS	High risk – needs a complete risk analysis		
Dicksonia antarctica	CA	Low risk – no need for a risk analysis		
	USA	Low risk – no need for a risk analysis		
	NZ	Medium risk – risk analysis needed, but not immediately		
Dryopteris erythrosora	CA	Low risk – no need for a risk analysis		
	USA	Medium risk – risk analysis needed, but not immediately		
	UK & RI	Low risk – no need for a risk analysis		
	AUS	Low risk – no need for a risk analysis		
	NZ	Low risk – no need for a risk analysis		
Lygodium japonicum	CA	Low risk – no need for a risk analysis		
Phlebodium aureum	NZ	Medium risk – risk analysis needed, but not immediately		
Sphaeropteris cooperi	USA	Low risk – no need for a risk analysis		
	UK & RI	Low risk – no need for a risk analysis		

Table 2). A. raddianum, however, was the only species categorised as high risk for the countries in which it is traded. Suitable environments are available for this species in two of the countries in which it is traded (the USA and Australia), and given that the species has had negative impacts in its alien range, it needs a complete risk analysis for these countries. D. antarctica and P. aureum were categorised as a medium risk for New Zealand, and D. erythrosora a medium risk for the USA (Table 6) as suitable environments are available, but these species have no documented negative impacts. These species require a full risk analysis in these countries, but resources should first be focussed on high risk species. The remaining cases (various species-trade country combinations, see Table 6) were categorised low risk, as there was no suitable environment predicted in the countries of trade. A full risk analysis is not currently required in these cases, but low risk species cannot be considered as 'safe' for trade due to their histories of invasion elsewhere (see Table 2). It should be noted that the models for S. cooperi and D. antarctica may be under predicting in the USA (Fig. 1), and so the indication that these species be considered a low risk for the USA should be treated with caution. For three species, A. raddianum, S. cooperi, and L. japonicum, suitable environments were predicted in some countries where the species are not yet known to occur. Further work is required in these regions in order to determine the risk posed, and whether risk analysis is required, especially considering that these species are particularly popular in trade and introduction through this pathway is likely.

Discussion

A broad environmental and climatic tolerance is exhibited in many popular horticultural species (Dehnen-Schmutz et al. 2007b; Hulme 2011) and thus is an attribute that is also known to promote invasion success (Hulme 2015; van Kleunen et al. 2018). As such, we expected the popular ornamental alien ferns assessed in this study to have wide environmental tolerances and, consequently, wide potential global distributions. However, the predicted distributions for most of these species are relatively limited (spanning few continents and countries), with only *A. raddianum, S. cooperi*, and *L. japonicum* showing wider potential global distributions (as compared to the other study species) both in regions where they are already known to occur and beyond their current known range of occurrence. It is unlikely that the relatively limited distributions predicted for most species are due to issues with model performance as the evaluation statistics indicated that the models performed well. Rather, this may be attributed to the habitat specificity commonly associated with fern species (Mehltreter 2008; Kessler 2010), which aligns with the suggestion that alien ferns are unlikely to invade to the extent noted for some prominent angiosperm invaders (Jones et al. 2020).

Despite the satisfactory performance of the models, a few of the response curves were truncated and slightly abnormal, indicating that the occurrence records of some of the assessed species might not characterise their full fundamental niche (i.e., the full set of conditions in which a species can survive in the absence of biotic interactions; Peterson et al. 2011) (Rodríguez et al. 2019; Soberón and Peterson 2020). This, however, is common, as the realised niche (i.e., the set of conditions in which the species occurs in the presence of biotic interactions, Peterson et al. 2011) often forms a subset of the fundamental niche; or because the occurrence records do not span the entire geographical range of the taxon (i.e., they incompletely characterise the realised niche). The latter circumstance, however, was avoided in this study as we compared the downloaded occurrence records with currently documented information on the range of each species to ensure, as far as is possible, that all areas occupied were accounted for in the models.

Variables that contributed highly to the models reflected well the documented biological and environmental limitations typical for most fern species. The large contribution of precipitation variables, for example, was unsurprising as ferns generally require moist environments (Ferrer-Castán and Vetaas 2005; Karst et al. 2005; Kessler 2010; Sharpe and Mehltreter 2010), and as precipitation plays a role in determining fern species richness (Lehmann et al. 2002; Bickford and Laffan 2006; Moreno Saiz and Lobo 2008; Nagalingum et al. 2015). The reliance of ferns on water is considered a limitation in their evolution (Page 2002), and most likely explains why terrestrial ferns are deemed unlikely to invade to the same extent as some of the world's most aggressive invaders (see Lowe et al. 2000; Jones et al. 2019). Ferns exhibit poorly controlled evapotranspiration which restricts the degree of exposure they can withstand and limits them to areas of high precipitation and humidity (Page 2002; Sharpe et al. 2010). Accordingly, <10 % of fern species in their sporophyte form (the distinct frond producing form) exhibit desiccation tolerance (Hietz 2010). Ferns rely on water to breed as sexual reproduction can only take place where free water is available to allow movement of the motile spermatozoids involved in fertilisation (Page 2002). In accordance with these requirements, precipitation of the driest quarter was, on average, an important predictor across species. Variables associated with temperature extremes also contributed to the models for some species, and in accordance with this, the majority of fern species cannot withstand frond freezing (Fernández-Marín et al. 2021). Mean temperature of the coldest guarter, for instance, was the most important predictor for A. raddianum and this tropical species generally selects for sheltered environments where frost is unlikely. In some instances, non-climatic variables (i.e., land cover) contributed most to the models and may be an indication of habitat specificity. For example, land cover was the most important predictor for *D. antarctica* and *P. aureum*, and these species displayed highly limited potential distributions in the models. Furthermore, in South Africa, D. antarctica has naturalised in the Cape Peninsula (Roux 2001; Crouch et al. 2011), but invasion has not yet occurred (Jones et al. 2020). This is suspected to be due to its slow growth rate and late age of reproductive maturity (~20 years) (Crouch et al. 2011), coupled with fragmentation of suitable forest habitat in the Western Cape (Roux 2001).

Although many ferns can colonise disturbed and altered habitats (Murakami et al. 2005; Walker and Sharpe 2010), typical fern habitat is characterised by wet, shady, undisturbed forests (Kessler 2010). For example, in their native range A. raddianum commonly favours shaded earth banks in forest (Crouch et al. 2011; Jones et al. 2020); P. aureum occurs in forest canopies, but also in the lower strata of the forest (Smith 1993); and *D. antarctica* is one of the dominant understorey species of wet evergreen forests (Ough and Murphy 1996). Similarly in their invaded range, alien ferns have generally been found to largely occur in sites associated with other invaders, close to water, and most often in forest systems or in an urban/forest matrix, for example in South Africa (Jones et al. 2020), Hawaii (Chau et al. 2013), and India (Morajkar et al. 2015). Although intact forests generally display some resistance to invasion (Fine 2002: Green et al. 2004: Levine et al. 2004), various shade-tolerant and often late successional species do show the capability to invade these environments (Martin et al. 2009). Ferns are a prime example of such species (Kessler 2010; Sharpe and Mehltreter 2010): in just over six years L. microphyllum (a climbing fern) formed dense mats covering large swathes of remote intact forests in Florida, USA (Volin et al. 2004). Similarly, the related climbing fern L. japonicum (modelled here) displays comparable behaviour where it invades in northern Florida (Lott et al. 2003). Some of the other alien ferns considered in this study have been shown to outcompete native species and change forest community structure in their invaded range. For example, D. antarctica has invaded two European Union conservation priority habitats on São Miguel Island where eradication is now deemed impossible (Arosa et al. 2012), and A. raddianum has replaced populations of the native fern Adiantum capillus-veneris L. in Hawaii, USA (Wilson 1996). It is their selection for, and evidence of impacts in some unaltered environments, such as forests, that warrants concern despite their relatively limited potential global extent for invasion.

The consideration of environmental suitability in conjunction with the invasion status of a species and the knowledge of their invasion and impact history has enabled us to classify the study species in terms of the invasion risk they pose in the countries in which they are traded, and thus get an indication of whether they require a complete risk analysis. The USA, Australia, and New Zealand show the greatest potential for invasions by these traded alien ferns, with suitable environments available for at least four of the six study species which now constitute medium or high risk species that require risk analysis. The USA is of particular concern, as this country already has extensive documentations of fern invasions and is a prominent trader in alien fern species (McCulloch-Jones et al. 2023). For example, in the USA, the Florida Exotic Pest Plant Council's 2019 List of Invasive Plant Species lists seven fern species as invaders that are changing community structures or ecological functions, or hybridizing with native species (FLEPPC 2019). Furthermore, in Georgia, USA, five alien ferns have recently been documented as spreading beyond their sites of introduction (Wyatt 2020), including D. erythrosora (modelled here) which was first noted outside of cultivation just 13 years ago (Simpson et al. 2008). According to our study, Georgia and its surrounding areas are environmentally suitable for this species, but the area available for range expansion is closely confined to its current invaded range (which spans a large area of south-eastern USA, excluding Florida). Similarly, New Zealand also has a history of multiple fern invasions (Brownsey 1980; Brownsey and Perrie 2014; Perrie et al. 2019) and there are suitable environments available in New Zealand for three of the study species, namely *D. antarctica*, *L. japonicum*, and *S. cooperi*, but these have a relatively limited potential range, and moderately suitable environments.

The USA, Australia, and New Zealand are most in need of trade regulations, specifically for the species identified as medium and high risk to prevent the occurrence of, or increase in, invasions. As official regulation is contingent on risk analysis, this study provides crucial information by identifying species that require risk analysis. Efforts to detect and manage escaped populations are necessary as suitable environments are available and invasive populations may remain undetected in these countries. Furthermore, countries that possess suitable environments, but lack documented occurrences of these species should conduct risk assessments. Based on the assessment outcomes, they can determine whether it is necessary to perform risk analysis and implement trade regulations to prevent future invasions. It is important to note that all species considered in this study are highly popular in trade and introduction via this pathway is thus likely (McCulloch-Jones et al. 2021). We suggest that key habitats (e.g., moist, shaded environments) that occur close to, or as a matrix with, urban areas are prioritised for efforts to locate undetected populations as this is where fern invasions most commonly occur. Trading countries for which the majority of species were categorised as a low risk (i.e., Canada and the United Kingdom and the Republic of Ireland) are under a lower potential threat of invasion and the current response arguably need not be as aggressive (i.e., risk analyses are not indicated, and trade bans are likely unnecessary). Importantly, the suggested categorisation of the study species should be revised periodically, given changes in land-use and climate, and as the occurrence and environmental data available for modelling could improve with time as further resources become available.

Conclusion

This study is the first to model the potential global distribution of multiple invasive alien fern species, identifying countries susceptible to invasion and informing the need for risk analyses. The models reveal relatively limited potential global distributions for these ferns compared to other major invaders, but their association with undisturbed habitats, such as forests, and their documented impacts in such habitats raises concern. The projections of environmental suitability have allowed us to complete risk assessments for the studied species to inform their requirement for risk analyses in the countries in which they are traded, thus initiating the early stages of management action. The USA, Australia, and New Zealand stand out as potential hotspots for invasion by traded alien ferns, necessitating management interventions and on-ground population detection for high or medium risk species. This risk assessment approach serves as a valuable management tool, highlighting focal species for each trade country and supporting efficient resource allocation in alien species management and regulation.

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Supplementary material I

Supplementary information

Authors: Emily J. McCulloch-Jones, Tineke Kraaij, Neil Crouch, Katelyn T. Faulkner Data type: docx

- Explanation note: (1): Comprehensive methods on the modelling procedure applied in the study. The sub-sections are divided as in the main text to improve readability; (2): The total number of occurrence records for each study species obtained from the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/) and the number of occurrence records available for modelling post-data cleaning; (3): A breakdown of the land cover variable which was acquired from the ESA CCI Land Cover project (http://maps.elie.ucl.ac.be/CCI/viewer/download.php). Left aligned numbers and land cover types are main categories, and right aligned numbers and land cover types are sub-categories; (4): Decision tree used for categorising the priority of species for risk analysis (after Bayón and Vilà 2019) in trading countries. All species are present in the trading countries considered and all are invasive somewhere in the world – hence 'yes' is the only possible response for the first two questions of the decision tree; (5): Response curves for the SDMs for each study species. Curves show how each environmental variable affects the Maxent prediction and how the predicted probability of presence changes each environmental variable is varied, keeping all other environmental variables at their average sample value.
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RESEARCH ARTICLE



Predation risk by largemouth bass modulates feeding functional responses of native and non-native crayfish

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Abstract

Context-dependency is prevalent in nature, challenging our understanding and prediction of the potential ecological impacts of non-native species (NNS). The presence of a top predator, for example, can modify the foraging behaviour of an intermediate consumer, by means of non-consumptive effects. This raises the question of whether the fear of predation might modulate consumption rates of NNS, thus shaping the magnitude of ecological impacts. Here, we quantified the functional feeding responses of three non-native crayfish species - red swamp crayfish Procambarus clarkii, rusty crayfish Faxonius rusticus and virile crayfish Faxonius virilis - compared to the native analogue signal crayfish Pacifastacus leniusculus, considering the predation risk imposed by a top fish predator, the globally invasive largemouth bass Micropterus salmoides. We applied the comparative functional response (FR) approach using snails as prey and exposing crayfish to water containing predator and dietary chemical cues or not. All crayfish species presented a destabilising Type II FR, regardless of the presence of chemical cues. Predation risk resulted in significantly longer handling times or lower attack rates in non-native crayfish; however, no significant differences were observed in signal crayfish. We estimated per capita impacts for each species using the functional response ratio (FRR; attack rate divided by handling time). The FRR metric was lower for all crayfish species when exposed to predation risk. Rusty crayfish demonstrated the highest FRR in the absence of chemical cues, followed by signal crayfish, virile crayfish and red swamp crayfish. By contrast, the FRR of signal crayfish was nearly twice that of rusty crayfish and virile crayfish and ten times greater than red swamp crayfish when chemical cues were present. The latter result agrees with the well-recognised ecological impacts of signal crayfish throughout its globally-introduced range. This study demonstrates the importance of considering the non-consumptive effects of predators when quantifying the ecological impacts of intermediate non-native consumers on prey. The direction and magnitude of the modulating effects of predators have clear implications for our understanding of NNS impacts and the prioritisation of management actions.

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Keywords

ecology of fear, decapods, higher-order predator, kairomones, trait-mediated indirect effects

Introduction

Non-native species (NNS) are a primary driver of environmental change, with negative impacts on individuals to entire ecosystems and severely disrupting important services provided by nature (Ricciardi et al. 2013). Economic burdens are also concerning, with estimated costs to prevent and control NNS impacts exceeding a hundred million dollars per year globally (Pyšek et al. 2020; Diagne et al. 2021), possibly increasing in the future (Ahmed et al. 2022). Management and policy strategies rely on identifying the most impactful species to help target prevention efforts and allocate limited resources to control or eradicate burgeoning populations. Still, this task is challenging due to variations in NNS impacts, based on ecosystem, geographical location, time since establishment and individual values (Závorka et al. 2018; Santos et al. 2019). Considering the increasing rate of invasions, comparative studies of the context-dependency of NNS impacts will help prioritise which species should be managed in the future (Lockwood et al. 2007; Dick et al. 2017a; Seebens et al. 2017).

Quantifying *per capita* effects of NNS remains central to most frameworks evaluating their ecological impacts (Parker et al. 1999; Kumschick et al. 2015; Griffen et al. 2020). Given the challenges of estimating *per capita* effects, focus has shifted to the use of experiments to quantify resource consumption rates as a proxy (Dick et al. 2017b). Non-native consumers often consume resources more efficiently than their native counterparts (Funk and Vitousek 2007; Salo et al. 2007; Paolucci et al. 2013), making comparative rates of consumption between native and NNS a useful currency to anticipate negative impacts from species introductions (Dick et al. 2014).

The fundamental ecological concept of functional responses (FR) – resource use as a function of availability – provides a measurable estimate of the *per capita* effect of a consumer on a given resource (Solomon 1949; Holling 1959; Dick et al. 2014). Type I FR describes a linear relationship between consumption and resource availability, typical of filter-feeding species (Jeschke et al. 2004). Type II FRs are destabilising due to high consumption rates at low resource densities, while Type III FRs promote stabilising effects due to low consumption rates when resources are scarce (Oaten and Murdoch 1975). The utility of FRs lies in comparing the maximum consumption rate between NNS and native trophic analogues in the same environmental context (Dick et al. 2014, 2017a), making this approach increasingly applied to quantify and predict ecological impacts of NNS (Faria et al. 2023).

The comparative FR approach enables the evaluation of *per capita* effects in different contexts, allowing more realistic and practical impact assessments (Dick et al. 2017a; Dickey et al. 2020). Despite this, investigations involving trophic levels beyond the focal consumer-resource interaction remain limited (e.g. Barrios-O'Neill et al. (2014); Paterson et al. (2015)). Foraging behaviour and consumption rates of consumers are sensitive to the presence of higher-order predators, which invoke tradeoffs in resource acquisition versus mortality risk by predation (Lima and Dill 1990; Brown and Kotler 2004). Fear of predation can shape entire ecosystems through traitmediated indirect effects (TMIEs) on prey traits, such as behaviour and physiology (Werner and Peacor 2003; Peckarsky et al. 2008; Laundré et al. 2010). In some circumstances, the non-consumptive effects of predators are thought to be as strong as direct consumption for population dynamics, leading to greater system stability (Brown et al. 1999) or even causing trophic cascades (Schmitz et al. 2004; Preisser et al. 2005; Peckarsky et al. 2008).

Trait-mediated indirect effects are particularly prominent in freshwater ecosystems, likely due to the effective transmission of visual and chemical cues indicating predator presence (Preisser et al. 2005). For example, the presence of largemouth bass (*Micropterus salmoides*) altered the foraging behaviour and habitat use of bluegill sunfish (*Lepomis macrochirus*) prey leading to cascading changes in zooplankton communities (Turner and Mittelbach 1990). In another example, rusty crayfish (*Faxonius rusticus*) displayed greater consumption of macrophytes when exposed to chemical cues from largemouth bass, suggesting a robust effect of predation risk on crayfish foraging behaviour (Wood et al. 2018).

Despite the strong effects of non-consumptive effects in shaping communities, they are relatively underexplored compared to consumptive effects in the context of quantifying NNS impacts. Applying the comparative FR approach, we aim to test whether the non-consumptive effects of a top predator, the non-native largemouth bass, mediate the consumptive impacts of three non-native crayfish species (*Procambarus clarkii, Faxonius virilis* and *F. rusticus*) and a native analogue (*Pacifastacus leniusculus*) preying on snails. We hypothesise that non-consumptive effects of a top predator will reduce consumption rates of all crayfish species, but to a lesser extent for non-native crayfish with a shorter evolutionary history with the predator. The differential response to predation risk imposed by the largemouth bass may explain the expected higher *per capita* effects of non-native consumers compared to native analogue species.

Methods

Study system

Our study system is a three-level food chain composed by a non-native top predator, the largemouth bass, an intermediate consumer represented by non-native or native crayfish (Table 1) and native freshwater snails (Gastropoda, Planorbidae) as the basal resource. Crayfish are known to be highly sensitive to different chemical cues such as predator odour, dietary and alarm cues (Beattie and Moore 2018; Wood et al. 2018; Wood and Moore 2020a, b) and these cues show utility in assessing TMIEs (Paterson et al. 2013). Thus, we used a combination of predator and dietary chemical cues to provide the biological context of predation risk in our comparative FR approach.

Table 1. Crayfish species examined in this study, including scientific and common names, history of introduction in the Pacific Northwest region, sampled populations (coordinates) and carapace length (CL) and mass, presented as the mean (SD), of the individuals used in the experiments.

Crayfish	Scientific name	Common name	Estimated time of introduction	Sampled population	CL (mm)	Mass (g)
	Pacifastacus	Signal crayfish	Native	Skykomish River, WA	50.2	36.9
A	leniusculus			(47.8482, -121.8403)	(4.3)	(10.3)
7	Duccambanic	Ped swamp	1970c	Pipe Lake W/A	53.6	30.7
	clarkii	cravfish	19708	(47.5907, -122.0389)	(5.7)	(13.0)
- Com	Faxonius rusticus	Rusty crayfish	2005	Magone Lake, OR (44.5486, -118.9119)	41.1 (3.1)	25.9 (5.3)
	Faxonius	Virile crayfish	1980s	Rattlesnake Lake, WA	46.0	32.6
-	virilis			(47.4308, -121.7715)	(3.5)	(7.6)

The geographic context of the study is the Pacific Northwest region of the US, where all species were sourced (Table 1). The apex predator largemouth bass has a native distribution that extends from north-eastern US to northern Mexico (Brown et al. 2009), with a long history of intentional introduction for recreational fishing in many regions of the US and the world, including the study region dating back to the beginning of the 20th century (Stein 1970). The non-native crayfish used in this study have a varied history of introductions in the region (Table 1) and are amongst the most widespread and harmful invasive crayfish species in the world (Twardochleb et al. 2013). Signal crayfish is the most widely distributed native crayfish species in the region (Larson and Olden 2011) and also highly invasive outside its native range (Usio et al. 2007; Twardochleb et al. 2013; Vaeßen and Hollert 2015). All crayfish species used in this study are omnivorous and nocturnal, presenting maximum feeding or growth rates between 20 and 30 °C (Crandall and Buhay 2008; Westhoff and Rosenberger 2016; Rodríguez Valido et al. 2021; Ruokonen and Karjalainen 2022).

Animal collection and maintenance

Largemouth bass were collected using electrofishing from Lake Washington, WA (47.6469, -122.2991) in October 2022. A total of 33 fish were captured and transported to the lab facility at the University of Washington, where they were maintained in a circular tank of approximately 800 l without shelter (hereafter stimulus tank), aerated and continuously filled with water from Lake Washington, in an open circulation system (mean total length = 194 mm, SD = 53). Fish were acclimatised to the stimulus tank for two weeks before the beginning of the trials.

A total of 433 crayfish were sampled using baited traps deployed overnight from lakes in Washington and Oregon States in October 2022 (Table 1). Crayfish were kept in tanks of 256 l separated by species, with a maximum stock density of 30 individuals per tank. Stock tanks were continuously filled with water from Lake Washington, in an open circulation system and contained abundant structure for shelter. Crayfish were fed daily with commercial algae pellets until satiation and were acclimatised for at least two weeks before being used in the experiment. Snails used as prey were obtained from various commercial pet retailers (shell length mean = 10.3 mm, SD = 2.4). Snails were kept in a separate tank, in the same conditions as crayfish.

Functional response experiments

Native and NNS of crayfish were tested for differences in their predatory rate of snail prey supplied in seven different initial densities (2, 4, 8, 12, 16, 24 and 40 snails) under the presence or absence of waterborne predator and dietary chemical cues (hereafter, predator treatment and control, respectively). Experiments were conducted in a fully-randomised design with respect to crayfish species and initial prey densities assigned to predator treatment and control. Experimental arenas were round opaque tanks (44.5 cm diameter, 42.5 cm height) filled with 10 l of water and no substrate or shelter were provided (Fig. 1A). At the predator treatment, water was supplied from the stimulus tank containing water from Lake Washington and bass (Fig. 1B), whereas under the control, just water from Lake Washington was supplied (as in the stock tanks) (Fig. 1C). Lake Washington water is piped directly from 10 m depth where largemouth bass and other fish species are at low abundance. Water was supplied continuously by dripping through small hoses (5 mm diameter) to ensure that chemical cues were present throughout the experiment (Fig. 1D). Water temperature in experimental arenas and stock tanks were similar, all demonstrating natural diel ranges of 12.5°–18.5 °C.

Fish were starved for a week and then fed every other day a diet of crayfish before and during the experiments. Small individuals of all crayfish species were supplied simultaneously until satiation to enhance the response of crayfish to conspecific dietary cues released by the fish (Beattie and Moore 2018; Wood et al. 2018). Crayfish were starved in a separate tank for 48 h before use in experiments to standardise hunger levels. After the starvation period, an individual crayfish was allocated to each



Figure 1. Experimental setup used in the functional response trials **A** experimental arena with a signal crayfish and snail prey during a trial **B** water from the tank containing largemouth bass (left) was pumped to a head tank (upper right) and **C** distributed to the experimental arenas in the predator treatment (upper-left row) or water was supplied directly from Lake Washington to the control arenas (lower-right row). Blue tanks in the background were stock and starvation tanks where crayfish were kept before being used in trials **D** the water from both treatments was supplied to each experimental arena via individually controlled hoses.

experimental arena containing one of the seven initial densities of prey and allowed to forage for 24 h. The number of remaining prey was recorded at the end of the trials, along with the number of attacked, but uneaten prey. Crayfish sex, carapace length (CL) and mass were recorded, as well as the water temperature at the end of the trial.

There were seven replicates for each combination of crayfish species, initial density of prey and treatment. At least five replicates of each combination were performed in the absence of crayfish to account for any background mortality of prey. Prey survivorship in these replicates was 99.9%, thus all prey deaths during experiments were attributed to crayfish predation. Crayfish were not reused in the experimental trials and replicates where crayfish moulted during the trial or one week after were repeated.

Data analysis

All statistical analyses were carried out in R version 4.1.2 (R Core Team 2021). Functional response analyses were conducted using the number of prey consumed as the response variable, under the frair package (Pritchard et al. 2017). For each crayfish species × treatment combination, FR type was determined by logistic regression of the proportion of prey consumed against initial prey density (Juliano 2001). If the proportion of prey consumed decreases with increasing prey density, it produces a significantly negative first-order term, indicating a Type II FR; if otherwise, a significantly positive first-order term is obtained then it indicates a Type III FR (Juliano 2001). When the results of the logistic regression were not conclusive, different FR models were fitted directly and compared using Akaike's Information Criterion (AIC) (Pritchard et al. 2017).

Based on these analyses, all FRs were then modelled as Type II. Maximum Likelihood model fitting was used to fit data to the Rogers' random predator equation (Rogers 1972) that accounts for the depletion of prey along the experimental duration:

$$N_e = N_0 (1 - \exp(a(N_e h - T)))$$

where N_e is the number of prey consumed, N_o is the initial density of prey, *a* is the attack rate, *h* is the handling time and *T* is the time available for predation in days (i.e. experimental duration). As N_e is obtained experimentally, the estimated FR parameters are attack rate and handling time, representing a measure of successful attacks and the time needed for a predator to handle and ingest a prey item, respectively. The Lambert W function is implemented to solve the fact that N_e appears on both sides of the equation (Bolker 2008).

To compare FR parameters a and h between predator treatment and control, we used the indicator variables method (Juliano 2001; Pritchard et al. 2017), as:

$$0 = N_0 - N_0 \exp\{[a + D_a(j)] \{[h + Dh(j)](N_e) - T\}\} - N_e$$

where *j* is an indicator variable that takes value 0 for control and 1 for predator treatment. The parameters Da and Dh estimate the differences between treatments in the value of the parameters *a* and *h*, respectively. If Da and Dh are significantly different from zero, then the estimated FR parameters differ between treatment and control (Juliano 2001). The maximum feeding rate of each crayfish species × treatment combination was calculated as 1/(hT) indicating the maximum number of prey that one crayfish can consume in one day (T = 1 day). Additionally, the functional response ratio (FRR) was calculated, as a comparative metric of the ecological impact of NNS (Cuthbert et al. 2019), dividing the attack rate parameter by the handling time (a/h). High values of FRR are indicative of strong *per capita* impacts, while low values indicate less impactful predators (Cuthbert et al. 2019).

Potential differences in the trial's water temperature amongst species and initial densities of prey were evaluated through Kruskal-Wallis tests, as well as differences in crayfish weight and carapace length (CL) amongst species and initial densities of prey. Water temperature in trials did not vary amongst species (Kruskal–Wallis $X^2(3) = 7.366$, p = 0.06) nor in association with the initial densities of prey tested (Kruskal–Wallis $X^2(6) = 11.339$, p = 0.08). Crayfish mass and carapace length varied amongst species (Mass: Kruskal–Wallis $X^2(3) = 96.73$, p < 0.001; CL: Kruskal–Wallis $X^2(3) = 224.73$, p < 0.001). The effect of crayfish size and sex on the proportion of prey consumed was investigated with Spearman's correlation and Mann-Whitney tests, respectively. Crayfish size (Mass: r = 0.01, p = 0.84; CL: r = -0.08, p = 0.12) and sex (Mann-Whitney U = 14828, p = 0.74) had no relation to the proportion of prey consumed.

Results

All crayfish species presented a destabilising Type II FR towards snail prey, regardless of the presence of chemical cues (Fig. 2). This was confirmed by the significantly negative first-order term of the logistic regression, except for red swamp crayfish under predator treatment and rusty crayfish under control, where the estimates were non-significant (Table 2). For these two specific cases, the FR type was determined by comparing the AIC of different model fittings. For red swamp crayfish, Type I had a lower AIC (Δ AIC = 0.8), while for rusty crayfish, Type II presented a better fit (Δ AIC = 1.1) compared to other models (generalised FR model, Type I and Type III).

Non-consumptive effects were observed for all non-native crayfish species, except for native signal crayfish (Fig. 2). Predation risk lowered the magnitude of the FR, which reflects reduced maximum consumption rates estimated in the predator treatment compared to the control (Table 2). These differences were driven by significantly longer handling time of snails for virile crayfish ($Dh = -0.03 \pm 0.008$, p < 0.001) and rusty crayfish ($Dh = -0.02 \pm 0.008$, p = 0.01) (Fig. 3A) and significantly lower attack rate displayed by red swamp crayfish ($Da = 0.25 \pm 0.09$, p = 0.006) (Fig. 3B) in the presence of chemical cues. No significant differences in handling time or attack rates were evidenced for signal crayfish between treatment and control.

Native signal crayfish demonstrated a greater consumption rate when exposed to predation risk compared to non-native crayfish (Fig. 2). *Per capita* effects of signal crayfish on snails, according to the FRR, was nearly twice that of rusty crayfish and virile crayfish and ten times greater than red swamp crayfish (Fig. 4, Table 2). By contrast, rusty crayfish demonstrated the highest FRR in the control, followed by signal crayfish, virile crayfish and red swamp crayfish (Fig. 4, Table 2).

Discussion

Predators can exert non-consumptive effects on prey that are comparable in magnitude to consumptive effects (Werner and Peacor 2003; Preisser et al. 2005). Despite that, non-consumptive effects remain largely under-studied in evaluations of ecological impacts of NNS (Sih et al. 2010). Here, we quantified rates of snail predation by multiple non-native and a native crayfish species in the presence or absence of chemical and dietary cues from a higher-order predator. We found that predation risk reduced maximum consumption rates of snails due to longer handling times or lower attack rates, but did not alter the shape of the FR curve. Reduced foraging activity is a common antipredator behavioural response of crayfish when exposed to predation risk (Gherardi et al. 2011b; Beattie and Moore 2018; Kenison et al. 2018). For example, red swamp crayfish significantly reduces the time spent feeding by adopting a lowered posture after being exposed to largemouth bass cues (Gherardi et al. 2011b).

Native signal crayfish was the only study species demonstrating little evidence for the effect of predation risk on the FR magnitude. This outcome is supported by a body



Figure 2. Functional responses of native and non-native crayfish feeding on snails under predator treatment and control **A** native signal crayfish **B** non-native red swamp crayfish **C** non-native rusty crayfish and **D** non-native virile crayfish. Lines represent model fit (solid line: predator treatment, dashed line: control). Points represent mean consumption and error bars represent \pm SE per density (filled circles: predator treatment, open circles: control; n = 7 per initial density × treatment combination).

of literature suggesting that the response of signal crayfish to predation risk is highly variable and context-dependent (Stebbing et al. 2010; Gherardi et al. 2011b; Beattie and Moore 2018; Wood and Moore 2020b). For instance, Stebbing et al. (2010) observed altered behaviour as raised posture in signal crayfish exposed to the chemical cues of European eels (*Anguilla anguilla*), but not to European perch (*Perca fluviatilis*), whereas Hirvonen et al. (2007) reported crayfish reduced shelter use when exposed to eel odour.

Treatment/ Species	1 st order term	FR	$a \pm SE$ (p-value)	$b \pm SE (p-value)$	1/ <i>b</i> T	FRR
	(p-value)	type				
Predator						
Signal crayfish (native)	-0.0617	II	2.26 ± 0.23	0.04 ± 0.004	28.02	63.5
	(> 0.001)*		(> 0.001)*	(> 0.001)*		
Red swamp crayfish	-0.0078	I†	0.25 ± 0.05	0.04 ± 0.037	24.81	6.1
	(0.29)		(> 0.001)*	(0.271)		
Rusty crayfish	-0.0283	II	1.16 ± 0.14	0.03 ± 0.006	31.37	36.3
	(> 0.001)*		(> 0.001)*	(> 0.001)*		
Virile crayfish	-0.0411	II	1.64 ± 0.23	0.05 ± 0.006	19.81	32.5
	(> 0.001)*		(> 0.001)*	(> 0.001)*		
Control						
Signal crayfish (native)	-0.0358	II	2.08 ± 0.24	0.03 ± 0.004	37.53	78.2
	(> 0.001)*		(> 0.001)*	(> 0.001)*		
Red swamp crayfish	-0.0142	II	0.50 ± 0.08	0.04 ± 0.015	28.59	14.3
	(0.022)*		(> 0.001)*	(0.021)*		
Rusty crayfish	-0.0101	II	1.14 ± 0.13	0.01 ± 0.006	94.99	108.0
	(0.086)		(> 0.001)*	(0.061)		
Virile crayfish	-0.0252	II	1.43 ± 0.15	0.02 ± 0.005	47.95	68.8
	(> 0.001)*		(> 0.001)*	(> 0.001)*		

Table 2. Functional response estimates of native and non-native crayfish species under predator treatment and control. The 1st order term of the logistic regression (see Methods), the functional response (FR) type, estimated parameters attack rate (*a*) and handling time (*b*), the maximum feeding rate (1/hT) and the functional response ratio (FRR). * = significant results.

[†]Despite being categorised as Type I using AIC, we fitted data to the Type II model to allow comparison of parameters between treatment and control.

These studies were performed in the invaded range of signal crayfish; thus, unexpected behavioural responses were attributed to naïve juvenile individuals with a lack of evolutionary history with these predators (Hirvonen et al. 2007; Stebbing et al. 2010). However, this may not be the case in our study where signal crayfish is native and has experience with largemouth bass in the region for over a hundred years. This suggests that signal crayfish may better assess the risk posed by a familiar predator using both chemical and visual cues (Blake and Hart 1993), whereas all three non-native crayfish species responded in a more conservative manner to the presence of chemical cues alone in a novel environment (Gherardi et al. 2002; Hazlett et al. 2003). Another possible mechanism is related to the larger body sizes of signal crayfish compared to other species (both in the wild and individuals used in this experiment: Table 1). There is evidence that crayfish can assess predator size through chemical cues (Wood and Moore 2020a; Wagner and Moore 2022); thus, the size of the largemouth bass in the experiment may have been too small relative to signal crayfish to elicit an antipredator behaviour resulting in reduced foraging rates.

All crayfish species presented a Type II FR, which is deemed to destabilise resource populations. This result aligns with the known impacts of these species on biomass and abundance of benthic invertebrates, particularly snails (Twardochleb et al. 2013). Moreover, when applying an FR-based metric to evaluate impacts, our native



Figure 3. Estimated functional response parameters of native and non-native crayfish species under predator treatment and control **A** handling time parameter *h* and **B** attack rate parameter *a*. Points represent the mean estimate of the model (filled circles: predator treatment, open circles: control) and error bars represent \pm SE. **p* < 0.1 and ***p* < 0.05.

comparator species generally showed a higher FRR than non-natives, which contradicts the pattern of invaders being more impactful than their native counterparts (Cuthbert et al. 2019). Nevertheless, signal crayfish is itself highly invasive in Europe, Japan and the south-western United States, usually reaching higher abundances than those observed in its native range (Larson and Olden 2013) and causing significant impact through their omnivorous feeding habits (Usio et al. 2009; Twardochleb et al. 2013; Vaeßen and Hollert 2015). Our results also align with a previous study that



Figure 4. The functional response ratio (FRR) of native and non-native crayfish species under predator treatment and control. The calculated FRR (a/h) is represented as bars (solid bars: predator treatment, shadowed bars: control) and error bars represent propagated standard errors of original estimates of parameters attack rate a and handling time h.

experimentally compared the predation rate of signal crayfish and non-native crayfish towards Chinese mystery snail (*Bellamya chinensis*), where native signal crayfish consumed significantly more snails of all size classes than did non-native crayfish (Olden et al. 2009). Indeed, previous studies that used the comparative FR approach to assess the impacts of signal crayfish where it is non-native found that the species generally present higher FR magnitude when compared to European native analogues, such as white-clawed crayfish (*Austropotamobius pallipes*) and noble crayfish (*Astacus astacus*), although impact varied with prey type (Haddaway et al. 2012; Rosewarne et al. 2016; Taylor and Dunn 2018; Chucholl and Chucholl 2021). Differences in experimental systems, such as diverse prey types and arena sizes, preclude us from comparing the native signal crayfish findings here to those of invasive populations of the species. Future comparative studies of the FRs of native and non-native populations are recommended.

We found significant differences amongst NNS predatory impacts towards prey. Rusty crayfish and virile crayfish showed consumption rates similar to those of native signal crayfish, whereas red swamp crayfish demonstrated the lowest feeding rate, despite the latter species being considered one of the most impactful invasive crayfish in the world (Lodge et al. 2012; Twardochleb et al. 2013). Even though all crayfish are considered omnivorous or polytrophic, there are marked differences in their predominant trophic ecology (Reynolds et al. 2013). For instance, red swamp crayfish has a lower trophic position than signal crayfish, which is consistent with the perceived impact on macrophyte communities of the former species (Matsuzaki et al. 2009; Larson et al. 2017). Similarly, Madzivanzira et al. (2021) also reported a lower FR magnitude of red swamp crayfish preying on catfish fry compared to a native analogue crab. Despite this, it has been demonstrated that invasive populations of red swamp crayfish in Europe present ontogenetic niche shifts and have opportunistic feeding habits, adjusting its diet to different biotic and abiotic contexts, which further explains its success as an invader (Correia 2002, 2003; Carreira et al. 2017; Jackson et al. 2017). Additionally, red swamp crayfish has weaker chelae closing force compared to other decapods, which helps explain its preference for feeding on softer resources (South et al. 2020). Our findings support previously observed impacts of rusty crayfish and virile crayfish on snail communities where they are invasive (Dorn and Wojdak 2004; McCarthy et al. 2006; Kreps et al. 2012). The greater consumption rate of invasive crayfish is likely associated with selected traits, such as boldness and foraging voracity in NNS populations, which are known to differ from their native range (Pintor and Sih 2009; Reisinger et al. 2017; Glon et al. 2018).

Previous studies that investigated TMIEs using the FR approach reported mixed outcomes. Considering simple habitats, the presence of predator cues reduced consumption rates of the amphipod *Echinogammarus marinus*, an intermediate predator, towards isopod prey (Alexander et al. 2013). By contrast, fish cues did not influence the FR's magnitude of two amphipod species (the native *Gammarus duebeni* and the invasive *Gammarus pulex*) towards three different invertebrate preys (Paterson et al. 2015). Our study reinforces the need for considering the wider biological context of ecological interactions when quantifying the impacts of NNS. Moving forward, we suggest three primary ways that future studies could further explore context-dependencies.

First, the effect of abiotic contexts, such as habitat complexity and presence of shelter, continues to be a research need. The Type II FR curves reported here align with general expectations from the broader literature (Jeschke et al. 2004); however, the lack of habitat complexity in experimental arenas may prevent the observation of more stabilising Type III responses (Alexander et al. 2012; Griffen 2021). Likewise, gravel substrate has been reported to reduce crayfish consumption of pelagic, but not benthic prey (Vollmer and Gall 2014; South et al. 2019). Additionally, shelter use is a common behavioural response of crayfish to predator cues (Blake and Hart 1993; Garvey et al. 1994) and could have further magnified the observed differences between the predator treatment and control reported in our study.

Second, it would be valuable to evaluate additional biotic contexts, such as alternative resource availability, the presence of intra- and inter-specific competitors and effects of visual predator cues. For instance, prey preference for different resources, such as macrophyte or detritus, could have a significant effect on FRs for omnivorous crayfish (Cuthbert et al. 2018; Médoc et al. 2018), ultimately defining their ecological impacts when invasive (South et al. 2019; Chucholl and Chucholl 2021). Better incorporating the effects of competitive interactions in FR experiments are also fundamental to more realistic scaling of NNS *per capita* effects in the wild (Dickey et al. 2020; Latombe et al. 2022). As crayfish can respond to predation risk using a variety of different cues, the relative importance of visual and alarm cues can also be investigated using FRs (Blake and Hart 1993; Ramberg-Pihl and Yurewicz 2020). Third, future research investigations discussed above would benefit from the linking of mesocosms experiments with in-situ field studies to ensure robust scaling of our understanding (Iacarella et al. 2018).

Conclusions

Ecological impacts of NNS are notoriously challenging to anticipate given a myriad of biotic and abiotic context-dependencies that can affect the organismal performance in nature. The comparative FR approach has been used to incorporate these context-dependencies to predict the impact of NNS, through relative comparisons of *per capita* effects (Dick et al. 2014; Cuthbert et al. 2019; Faria et al. 2023). Here we showed that the presence of a higher-order predator can alter important parameters of FR, with direct effects on maximum consumption rates and predicted impact of intermediate non-native consumers. These findings suggest that the broader biological context in which consumer activities take place should not be overlooked if we aim to understand the ecological impacts of NNS. Likewise, biogeographic origin alone is not the sole indicator of impact, as we found that native signal crayfish demonstrated the highest estimated impact on prey in the presence of predation risk by a fish predator.

The ecology of fear predicts that the cost of anti-predator behaviour is associated with reduced offspring, thus modulating consumer abundance (Zanette and Clinchy 2019). Given the immense challenges in eradicating and controlling invasive crayfish populations (Gherardi et al. 2011a; Manfrin et al. 2019), this raises the interesting question of whether chemical cues could be used as an additional management tool to reduce their short-term ecological impacts, while other control strategies are being implemented. We encourage more research on which and how chemical components of predator and dietary cues trigger behavioural responses in crayfish, as these are not entirely elucidated (Mitchell et al. 2017), but have potential management applications.

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Supplementary material I

Data from functional response trials

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Data type: xls

- Explanation note: Data from functional response trials for each crayfish species under predator treatment and control.
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