

Research Article

A population dynamics approach to understand the invasiveness of the seaweed *Rugulopteryx okamurai* (Ochrophyta, Dictyotales)

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Abstract

The success of invasive species can be measured by invasiveness, which depicts intrinsic characteristics that enable them to thrive in new environments. In invasive seaweeds, for example, the persistence of multiple overlapping cohorts throughout the year plays a key role in increasing plant cover and exerting unrelenting pressure on invaded areas. The marine brown macroalgae *Rugulopteryx okamurai* has recently established abundant populations in the Mediterranean Sea and Atlantic Ocean, negatively affecting both biodiversity and socioeconomic factors by unprecedentedly aggressive invasive behaviour. The objective of the study is to understand the invasiveness of *R. okamurai* through its population dynamics. For this, a year-round study was conducted in a protected habitat of *Posidonia oceanica* in southern Spain, revealing that *R. okamurai* uses alternating mechanisms for population maintenance. It achieves high density of young individuals in late summer and autumn, peaking at 3285 individuals per square metre. In spring and early summer, the population shifts towards fewer – but larger – individuals, with densities dropping to 888 individuals per square metre and biomass reaching a peak of 170 g dry weight (DW) per square metre. Six overlapping cohorts were identified by Gaussian curves. They persisted throughout the year, but they were not related to environmental factors, which indicates adaptive physiological mechanisms that sustain dense monospecific populations. Additionally, the association between cohorts and different morphotypes suggests that *R. okamurai* phenotypic plasticity enables its persistence in introduced areas. These findings provide valuable insights into the biological traits underpinning its invasiveness in *P. oceanica* meadows, revealing temporal windows of invasiveness driven by different mechanisms. This knowledge is crucial for developing effective conservation and management strategies aimed at mitigating the impact of this invasive species.

Key words: Demography, density, generation, macroalga, *Posidonia oceanica*, recruitment



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Introduction

Invasion success refers to an invasive species' ability to progress through all stages of invasion, from introduction to dispersion, by overcoming ecological barriers and progressing through successive stages of establishment and expansion in a new environment (Gioria et al. 2023). Studies aiming to identify and understand the factors that contribute to the success of invasive species have led to the development of various invasive hypotheses (Lowry et al. 2013). Currently, there are

more than thirty invasion hypotheses that try to explain the relationship between invasive species, invaded communities and their interactions (Enders et al. 2018). According to Gioria et al. (2023), invasion hypotheses can be grouped into three major categories, namely: (1) propagule pressure, which involves the introduction efforts by invasive species in a new area and is defined as the number and frequency of propagule release (Lockwood et al. 2005; Colautti et al. 2006), (2) invasibility, referring to the characteristics of the receiving ecosystem and its susceptibility to being invaded (Catford et al. 2012; Colautti et al. 2014) and (3) invasiveness, referring to intrinsic characteristics of the species, including functional traits that determine its invasive potential (Pyšek et al. 2014; Jehangir et al. 2024). In this context, identifying the traits that serve as good predictors of invasiveness involves several factors, including physical performance, growth rate, size, biomass allocation, physiology and phenotypic plasticity (Richardson and Pyšek 2006). The latter expands the range of responses to biological and abiotic factors encountered in introduced areas, allowing the species to adapt quickly to the new environment and facilitating their establishment and expansion (Richards et al. 2006). This trait has been used to evaluate invasiveness by comparing population abundances and survival rates with native populations (Ebeling et al. 2008; Colautti et al. 2014). Collecting censuses across all major life stages of invasive species and employing demographic models are essential tools for gaining deeper insights into the population dynamics of invasive species (Williams et al. 2010; Colautti et al. 2014).

The study of seaweeds' invasiveness has predominantly focused on a limited number of species (Dalla Vecchia et al. 2020). The common traits that contribute to their invasiveness are morphology, physiologic plasticity, productivity and reproduction. These are the most studied characteristics (Mabey et al. 2023). Invasive seaweeds often show rapid growth rates and early maturation of reproductive structures, which allows them to quickly establish and spread in new environments (South et al. 2017; Zanolla et al. 2017). Other traits, including multiple overlapping cohorts maintained by both older individuals and new recruits, were described for *Undaria pinnatifida* (Harvey) Suringar (Schiel and Thompson 2012) and *Asparagopsis taxiformis* (Delile) Trevisan (Zanolla et al. 2019). This ability to persist year-round in introduced areas has been attributed to their morphological and physiological plasticity (Campbell 1999; Zanolla et al. 2015).

Rugulopteryx okamurae (E.Y. Dawson) I.K. Hwang, W.J. Lee & H.S. Kim (Dictyotaceae, Phaeophyceae), a flattened brown seaweed with dichotomous branching (Hwang et al. 2009), is native to the western Pacific Ocean (Verlaque et al. 2009). Starting as an unprecedented cryptic invasion in the western Mediterranean in 2015, it now forms abundant populations in Spain, including the Canary Islands and the Chafarinas Archipelago (Altamirano et al. 2016; REDEXOS 2022); France (Ruitton et al. 2021); Italy (Bellissimo et al. 2024); Morocco (El Aamri et al. 2018); and Portugal, including the Azores and Madeira (Faria et al. 2021; Bernal-Ibáñez et al. 2022; Liulea et al. 2023). Currently, *R. okamurae* continues its expansion both in the Mediterranean Sea and the Atlantic Ocean, being most recently recorded in northern Spain, which indicates a worrying upward trend in its spread (Díaz-Tapia et al. 2024). Predictive models suggest that the European and north African coasts are highly favourable habitats for this species, so it is likely that its expansion will continue in the coming years (Muñoz et al. 2019). Like other Dictyotaceae species, *R. okamurae* exhibits a digenetic isomorphic life cycle, alternating haploid gametophytes with diploid sporophytes. At a morphological

level, it presents up to three different morphotypes throughout the year in both native and introduced areas (Sun et al. 2006; Salido and Altamirano 2020). The species can reproduce by propagules, asexually by mitotic monospores and sexually by gametes and meiotic tetraspores, but gametangia have only recently been observed in northern Spain (Díaz-Tapia et al. 2024).

Rugulopteryx okamurae can settle on both horizontal and vertical rocky surfaces within a bathymetric range from eulittoral zones to depths of more than 50 m (García-Gómez et al. 2018; Altamirano et al. 2019). In southern Spain, *R. okamurae* has seriously impacted native flora, including the seagrass *Posidonia oceanica* (Linnaeus) Delille (García-Gómez et al. 2018; Junta de Andalucía 2019). *Posidonia oceanica* is a native seagrass listed in the Spanish List of Wild Species under the Special Protection Regime (*Listado Español de Especies Silvestres en Régimen de Protección Especial y Catálogo Español de Especies Amenazadas*) (Real Decreto 139/2011). It is also considered to form a priority habitat by the Habitats Directive (Council Directive 92/43/EEC). Similar impacts on native flora have been observed in the Azores Islands (Faria et al. 2022), where *R. okamurae* is replacing dominant species and altering the structure of shallow-water benthic communities. The fast and abundant proliferation of *R. okamurae* biomass not only produces high environmental impacts, but also socioeconomic ones (Altamirano et al. 2016; MITECO 2022). In particular, the fishing industry has been suffering economic losses estimated in millions of euros because of a decrease in fish stocks (MITECO 2022). Moreover, the extensive efforts to remove castaway biomass accumulated from beaches in tourist areas is also affecting local administrations (MITECO 2022). All these impacts have led to its inclusion in the List of Invasive Alien Species of Union Concern, being the first seaweed to be added (Commission Implementing Regulation (EU) 2022/1203 of 12 July 2022 amending Implementing Regulation (EU) 2016/1141). However, despite numerous studies about the species' potential biotechnological uses (e.g. Romero-Vargas et al. (2024)), only few studies focused on its basic biology, which is key to our understanding of the risks associated with its invasion and to lay the groundwork for implementing effective conservation and management strategies for affected marine ecosystems.

In this context, the objective of the present study is to understand the invasiveness of *R. okamurae* by analysing its population dynamics on an invaded *P. oceanica* meadow in southern Spain and exploring the relationship of several demographic descriptors with abiotic environmental factors throughout the year.

Methods

Study area and sampling procedure

The study was conducted on a population of *R. okamurae* established on a *P. oceanica* meadow located in front of Cambriles Cliff, Granada, Spain (36°44.0033'N, 3°20.6767'W), at a depth of 10 metres (Fig. 1). This *P. oceanica* meadow is 48 hectares in extent, ranging from 7 metres to 13 metres in depth (Portal Ambiental de Andalucía 2024).

Sampling for the study was carried out by scuba every two months from July 2021 to July 2022. Due to the protected status of the *P. oceanica* habitat (Real Decreto 139/2011) and the status of the invasive species *R. okamurae*, appropriate permits were obtained from the relevant authorities. Prior to the study, the minimal sam-

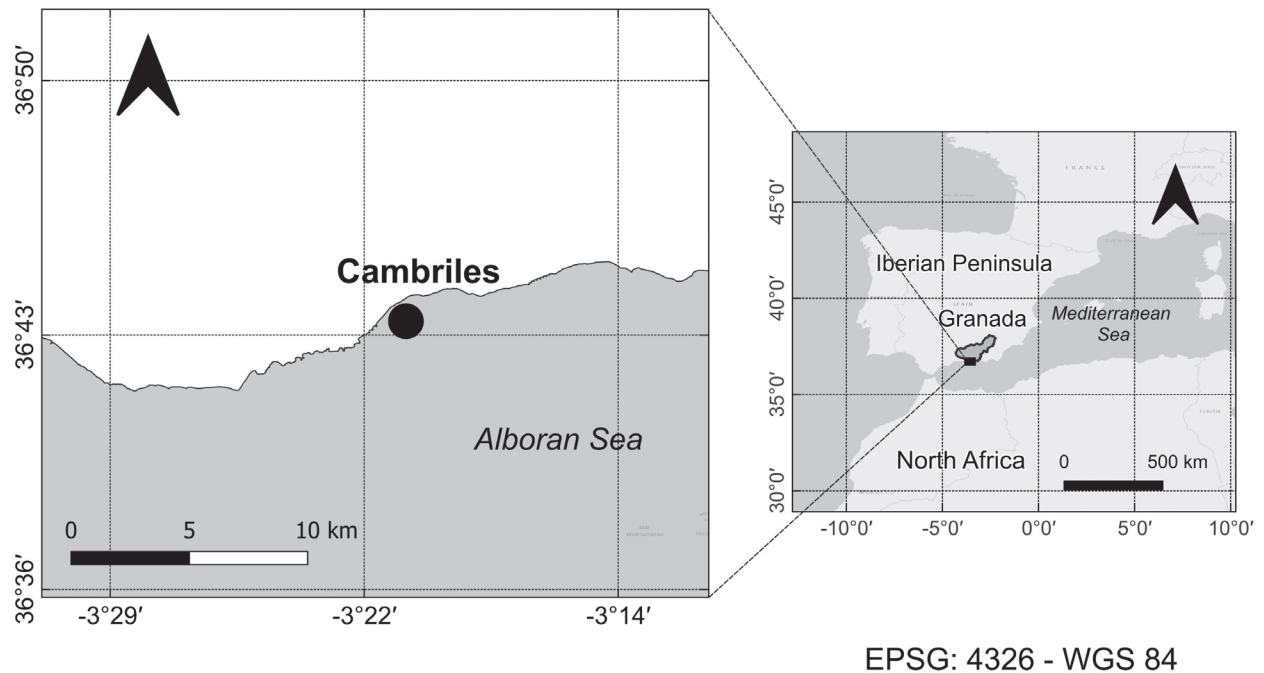


Figure 1. Sampling site of *R. okamurae* in Cambriles cliff, Granada (Spain).

pling area of *R. okamurae* was estimated following Cain and Castro (1959). All algal material in three one-metre by one-metre quadrats, each subdivided into 20 cm × 20 cm squares and spaced five metres apart, was carefully scraped and bagged separately in plastic bags. Samples were transported to the laboratory for weighing and for estimating the minimal sampling area through iterative measurements of contiguous squares. The process involved analysing the abundance data to estimate the smallest square size that showed no statistical differences in abundance between contiguous squares and through the estimation of the aggregation index, based on the ratio of variance to mean biomass, as described by Blackmann (1942). The minimum sampling area was 30 cm × 30 cm. Thus, within three areas with a homogeneous biomass density of *R. okamurae*, four replicates of the minimal area were taken and collected at the same depth, separated by 5 m between sampling areas. All algal material was thoroughly removed avoiding damaging *P. oceanica* plants and placed in plastic bags, preventing accidental dispersal of *R. okamurae*. Subsequently, the samples were transported to the laboratory under cold and dark conditions for further analysis.

Population dynamics

All individuals within each sample were measured from the base of the thallus to the most distal dichotomy and categorised into eight different size classes in two-centimetre increments, except the first class (four centimetres), which corresponds to young individuals or recruits. The abundance of *R. okamurae* was estimated as the number of individuals and their dry weight (DW) biomass per square metre for each sampling event. Dry weight biomass was quantified after drying at 60 °C for 48 hours in an oven. Distribution frequency of size classes plots was constructed using the percentage of individuals in each size class against the total number of individuals in each replicate and the mean values calculated. Cohorts, defined as individuals sharing a particular event during their lifespan (Crisp 1971), were

identified by tracking the displacement of Gaussian curves in the frequency versus time histograms, based on distributions of size classes along the year (Aranda et al. 1984; Zanolle et al. 2019). If more than one cohort overlapped within a given sampling time, a separation was achieved through a numerical fit using Gaussian curves (Petersen 1912; Zanolle et al. 2019). Each cohort was represented using all replicates by a Gaussian curve characterised by its mean and standard deviation (SD) through the Distr. Norm function. The adjustment of these parameters was performed using the SOLVER application (Excel, Windows Office 365). Finally, a statistical comparison of each mean and SD from the curves was carried out using a t-test statistical analysis. If no statistical differences were found, we assumed the existence of a single cohort. The model's significance was validated through R^2 .

Environmental factors

Daily data on average temperature, maximum and minimum temperature and salinity from a buoy situated near the study area were obtained from Puertos del Estado website (Ministerio de Transportes y Agenda Urbana), (SIMAR 2044080; 36.67°N, 3.5°E). The photoperiod was provided by the Observatorio Astronómico Nacional website, located in Granada (Instituto Geográfico Nacional, Ministerio de Fomento). In addition, three seawater samples were collected on each sampling date at the same depth as the *R. okamurae* population and analysed using an automated nutrient analyser QuAatro AQ2 AACE (Seal Analytical Ltd. Fareham, UK) for ammonium (Slawyk and MacIsaac 1972), nitrate and nitrite (Shinn 1941; Wood et al. 1967) and phosphate (Murphy and Riley 1962) following standard procedures.

Statistical analysis

Each biological variable was analysed using a one-way model ANOVA ($P < 0.05$), with time as a fixed factor. Homoscedasticity and normality were tested prior to the ANOVA by Levene's and Saphiro-Wilks test, respectively. When significant differences were found for a given biological variable, the Student-Newman-Keuls test (SNK) was applied for post hoc comparisons. Statistical analyses of ANOVA were carried out by SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA). To assess the relationship between environmental factors and biological variables, a principal component analysis (PCA) was conducted. An environmental space was constructed with axes derived from PCA using environmental factors and plotted in InfoStat version 2008 (Di Rienzo et al. 2008). Subsequently, Pearson correlations were used to investigate the relationship between biotic variables (density of individuals and biomass of *R. okamurae*) and the two principal axes derived from PCA (PC 1 and PC 2). These environmental axes produced in PCA were computed with the software PAST (Hammer et al. 2001).

Results

Population dynamics

Rugulopteryx okamurae was present throughout the whole year, displaying significant differences (DF: 6, F-value = 6.5, $P = 0.002$, Appendix 1) in density of individuals for different months (Fig. 2A). The highest values were recorded in September and No-

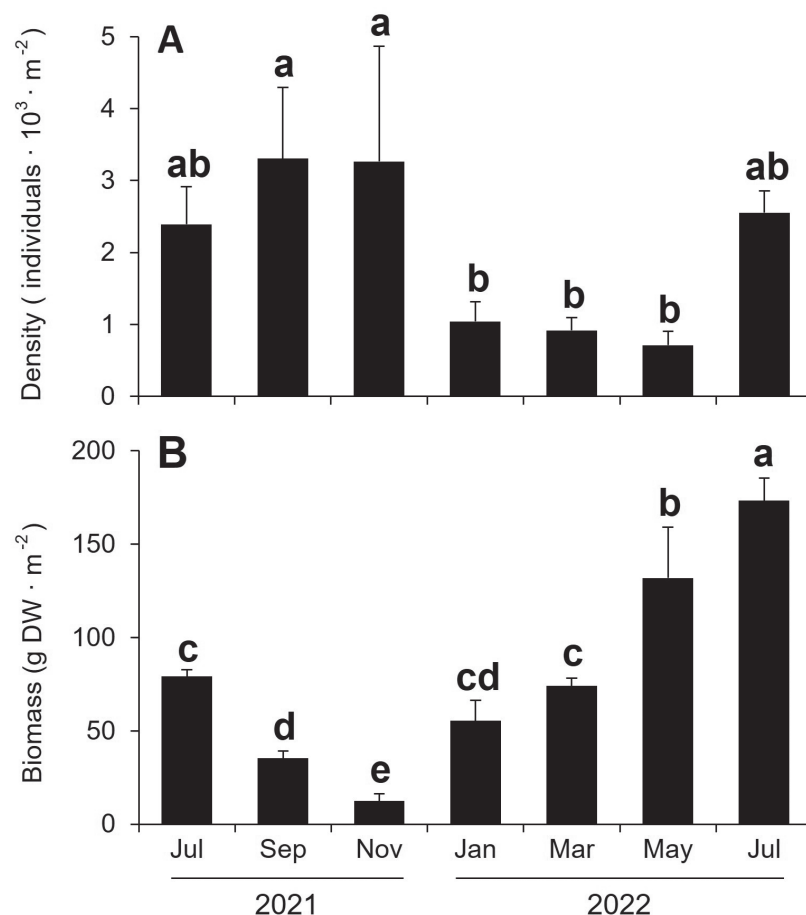


Figure 2. Density of *R. okamurae* throughout the study period (July 2021–July 2022) referred as **A** individuals (10^3 individuals per square metre) and **B** biomass (g DW per square metre). Data are expressed as mean \pm SD ($n = 3$). Different letters denote significant differences among months following ANOVA results ($P < 0.05$).

vember, with an average value of 3285 individuals per square metre, whereas the lowest values were observed in January, March and May, with a decrease of more than one-third (888 individuals per square metre) (Fig. 2A). There were also significant monthly differences in biomass (Fig. 2B, DF: 6, F-value = 60.6, $P < 0.001$, Appendix 1). These values increased from November to July 2022, when they were 14 times higher.

The distribution of size classes varied throughout the year, reaching 18 cm in May and July 2022, representing less than 3% of the total individuals in those months. In contrast, in November, individuals did not exceed six centimetres (Figs 3, 4). Over 70% of the total population belonged to the size class of 0–4 cm in September and November (Figs 3, 4). However, there was an 85% reduction in the density of the smallest size class from November to January and it continued to decrease until May, when the lowest numbers were recorded (Figs 3, 4).

Cohorts

The frequency distribution of thallus size classes followed a normal distribution, revealing the presence of six cohorts (named using Roman numerals I to VI) with different longevity and temporal distribution (Fig. 4). The longest cohort started in

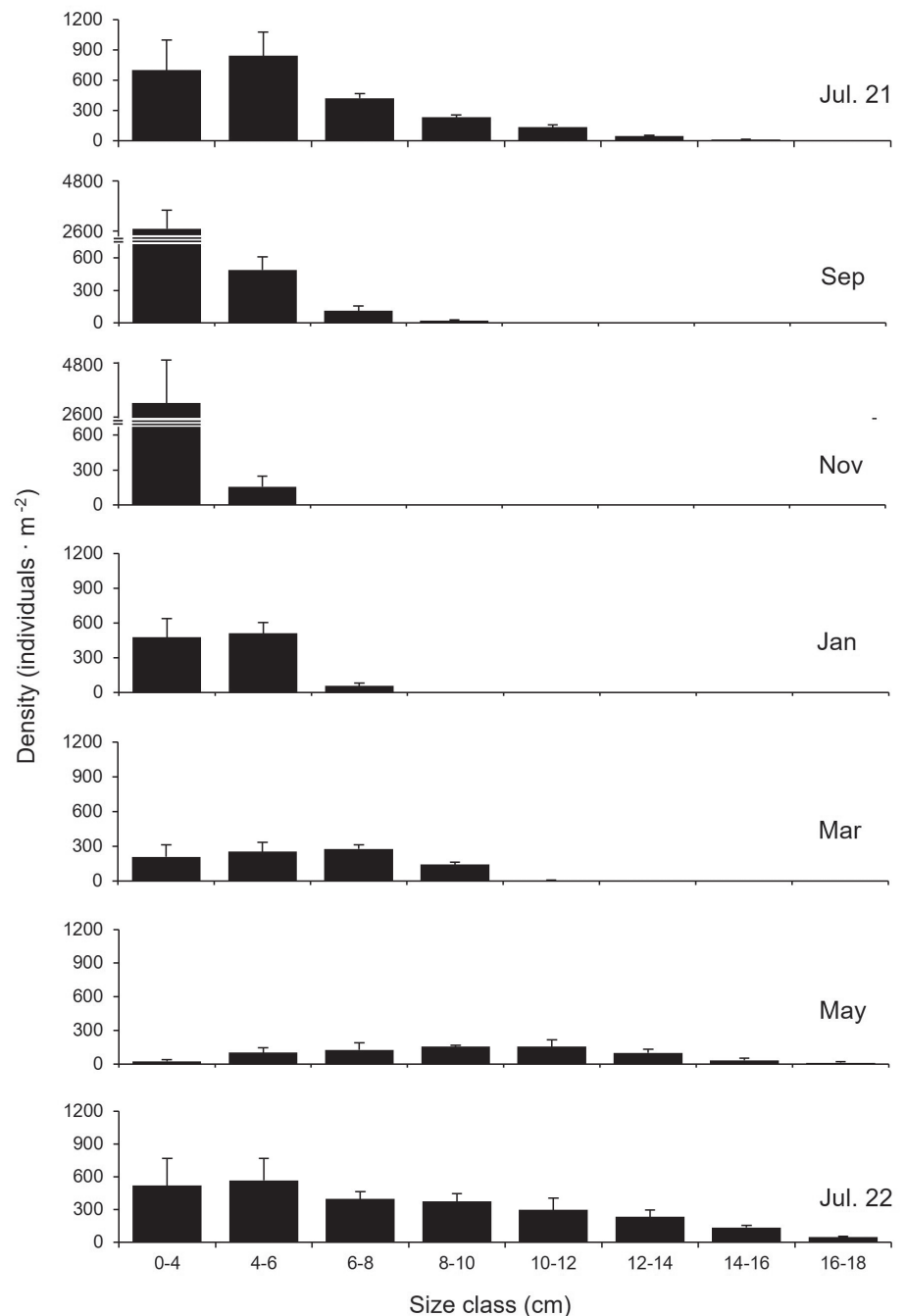


Figure 3. Density (individuals per square metre) of each size class throughout the study period (July 2021 to July 2022). Data are expressed as mean \pm SD ($n = 3$).

November and persisted until May (cohort IV), while cohorts I, III, V and VI had a lifespan of one month each (Fig. 4). Cohort II was present in July and September 2021. The composition of these cohorts also showed variations (Fig. 4). Cohort III was composed solely of young individuals, whereas cohorts IV and VI included individuals from different size classes, including individuals up to 18 cm (Fig. 4). However, it took six months for plants in cohort IV to achieve the largest size class, but only two months for those in cohort VI (Fig. 4). Additionally, overlapping cohorts were observed in July 2021 (cohorts I–II), September (II–III) and May (V–VI) (Fig. 4).

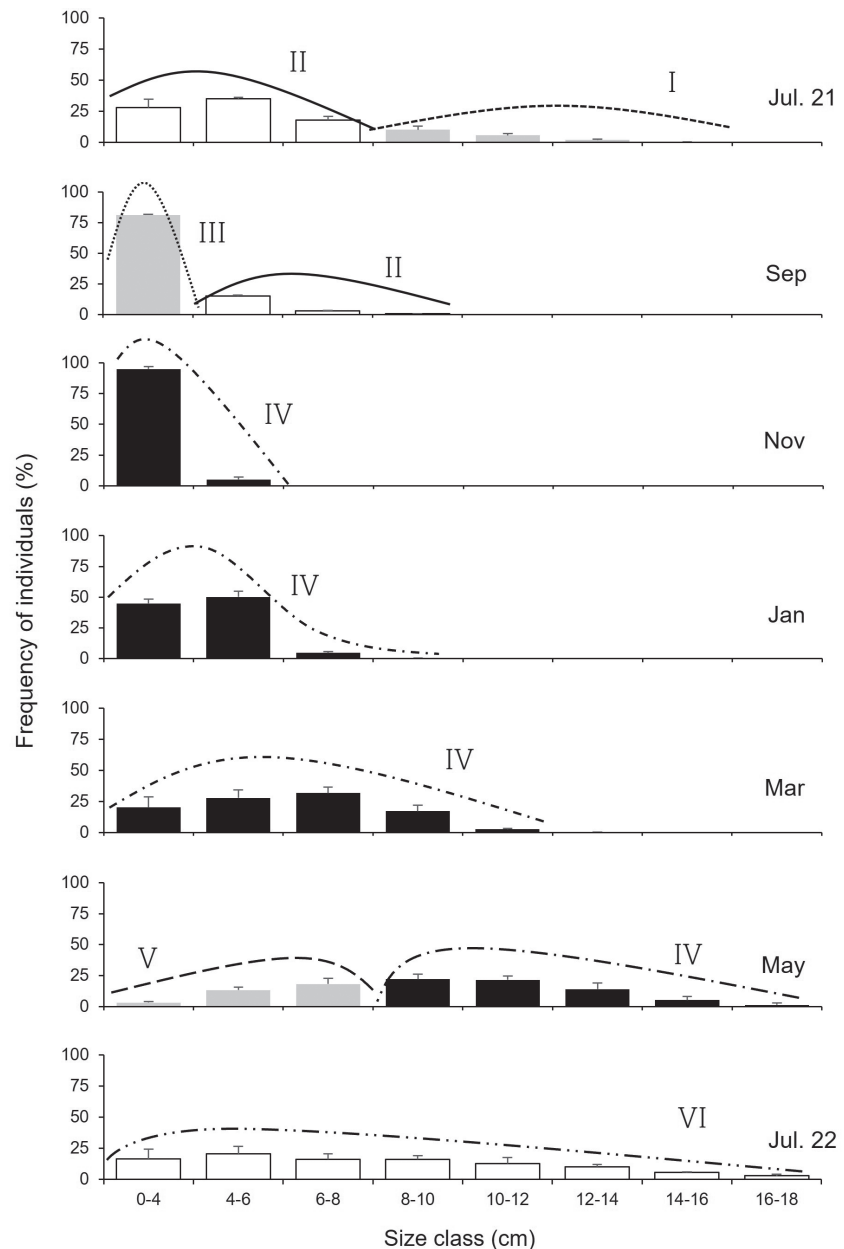


Figure 4. Frequency of individuals of each size class (%) related to the total of individuals of *R. okamurae* in each sampling event. Discontinuous lines indicate the six cohorts (labelled I to VI) found throughout the study (July 2021–July 2022). Values are expressed as means \pm SD ($n = 3$).

Environmental factors

Average temperature ranged from 15 °C to 23 °C, with the maximum temperature registered in August 2021 (25.1 °C) and the minimum (14.8 °C) in January (Fig. 5A). Low variability (> 1 °C) was registered in the winter months but, in summer 2021, it reached a difference of 8 °C (Fig. 5A). The photoperiod varied within a range of 10 to 14 hours (Fig. 5B). Salinity remained relatively stable during the study period, ranging from 36.8 to 37.3 (Fig. 5B).

Nitrate concentration during the study period ranged from < 0.2 μ M (the detection limit of the analytical method) to 1.4 μ M (Fig. 6). Ammonium concentration did not display significant seasonal variations, oscillating between 0.5 and 1.6 μ M (Fig. 6). Concentrations of nitrite and phosphate were too low to be detected (< 0.2 μ M).

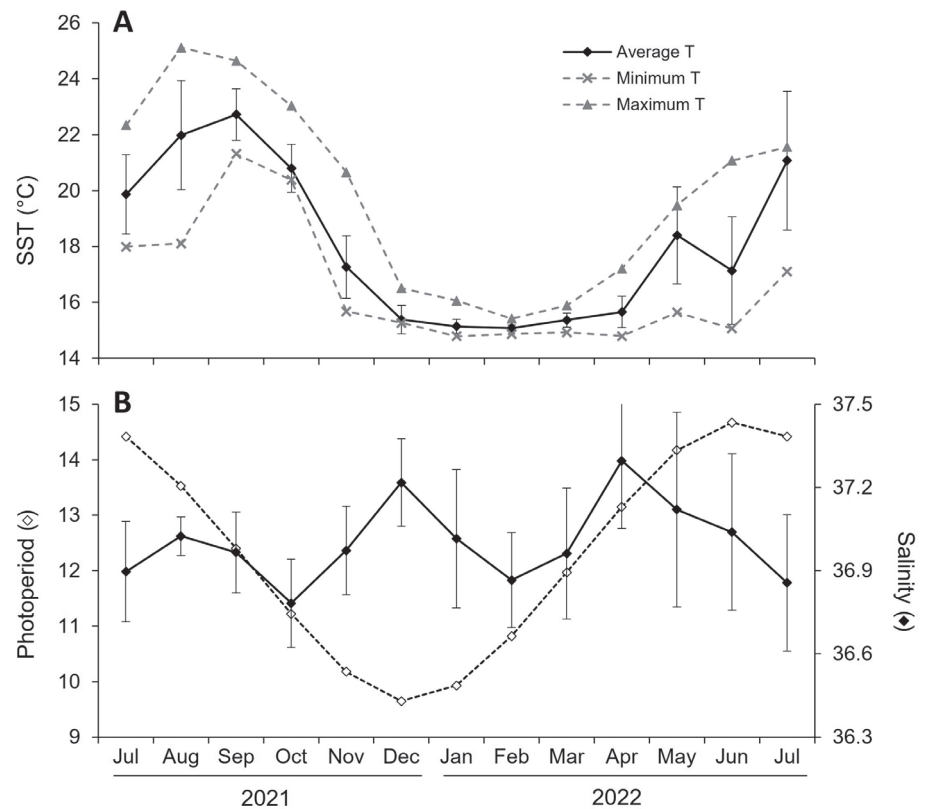


Figure 5. Environmental factors during the study period (July 2021–July 2022) in the study area (buoy SIMAR 2044080) **A** average data of mean, maximum and minimum temperature **B** monthly average photoperiod (dotted line) and salinity levels (solid line). Data expressed as mean \pm SD of daily data.

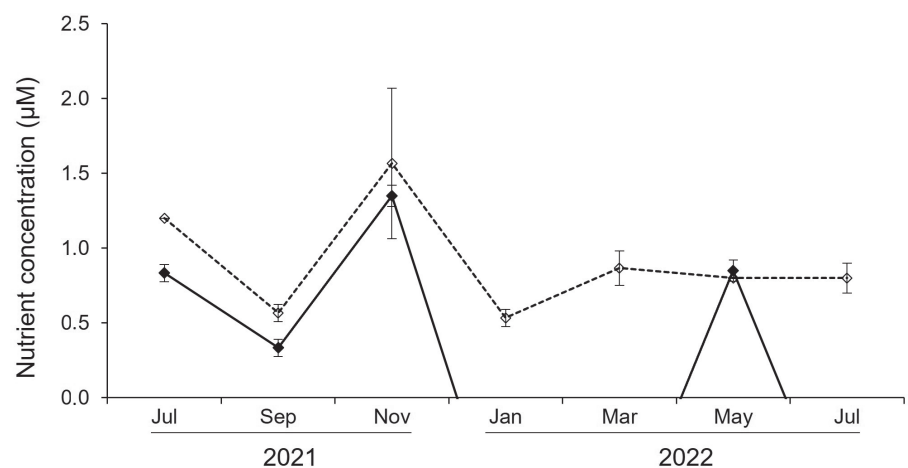


Figure 6. Nitrate (solid line) and ammonium (dotted line) concentration during the study period (July 2021–July 2022) in the study area. Data expressed as mean \pm SD ($n = 3$).

Principal component analysis (PCA) of environmental factors indicated that specific abiotic factors contributed differently along the two main axes (PC 1 and PC 2), explaining 79.8% of the total variance (Fig. 7, Appendix 2). Temperature, photoperiod and salinity contributed mainly for the PC 1, while nitrate and ammonium concentrations were the primary factors contributing for the PC 2 (Fig. 7). Pearson analysis between biological variables (density of individuals and biomass) and the two principal components derived from the PCA showed no significant correlations (P values > 0.05 , Appendix 3).

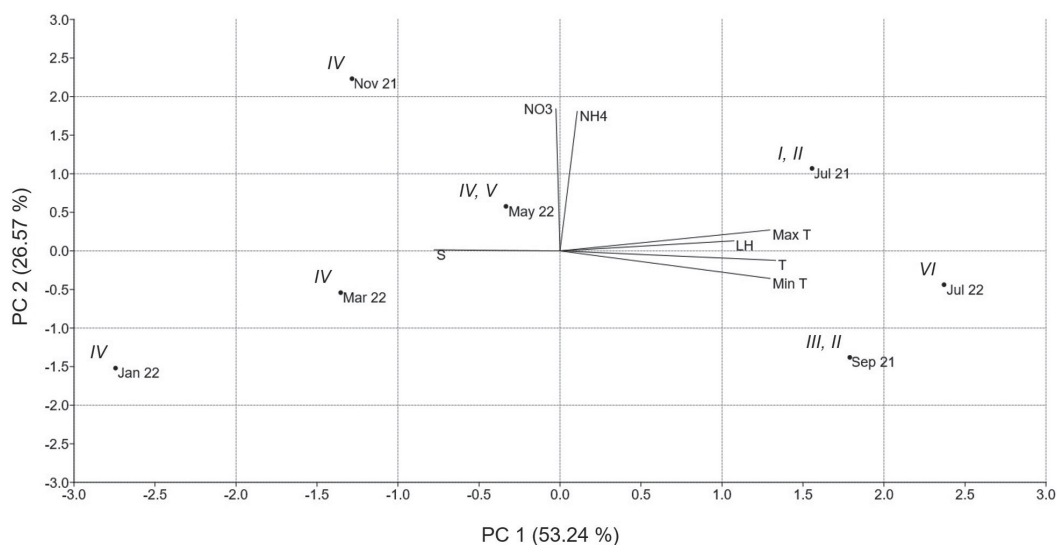


Figure 7. Environmental space derived from PCA constructed by environmental factors measured in the study area. Cohorts were labelled as I to VI. T, average temperature; Max T, maximum average temperature; Min T, minimum average temperature; S, salinity; LH, Photo-period; NO₃⁻, concentration of nitrate; NH₄⁺, concentration of ammonium; PC, principal component.

Discussion

Population dynamics of *R. okamurae* on a *P. oceanica* meadow during the year explains its invasive behaviour, characterised by the constant presence of a high density of individuals. This is achieved through its ability to continuously produce recruits and the presence of year-round short-lived overlapping cohorts that are not affected by the environmental parameters of the area.

The seaweeds' continuous presence is a characteristic of invasiveness shared amongst invasive seaweed species in invaded areas. Examples include the red seaweeds *Womersleyella setacea* (Hollenberg) R.E. Norris (Cebrian and Rodríguez-Prieto 2012), *Asparagopsis armata* Harvey (Aranda et al. 1984) and *A. taxiformis* (Zanolla et al. 2019), the brown seaweeds *Sargassum muticum* (Yendo) Fensholt (Thomsen et al. 2006) and *U. pinnatifida* (Schiel and Thompson 2012) and the green seaweed genus *Caulerpa*, specifically *C. taxifolia* (M. Vahl) C. Agardh (de Villèle and Verlaque 1995) and *C. cylindracea* Sonder (Ruitton et al. 2005). All these species showed the ability to maintain stable populations throughout the year, suggesting the existence of adaptive physiological mechanisms to the newly-invaded area and posing a continuous impact over native communities. Likewise, *R. okamurae* demonstrated a remarkable ability to establish dense monospecific populations, maintaining densities exceeding 3000 individuals per square metre and reaching biomass up to 170 g DW per square metre. These values greatly surpass other documented invasive seaweeds. For instance, *A. taxiformis* in the southern Iberian Peninsula, reached the highest biomass in the summer (Zanolla et al. 2017), but these values were 40% lower than those recorded for *R. okamurae* in this study. Another example was *U. pinnatifida* with a maximum density of about 100 individuals per square metre in affected areas in New Zealand (Schiel and Thompson 2012) or *S. muticum* with a maximum of 1000 individuals per square metre in Spain (Arenas and Fernández 2000). These are 97% and 67% lower than for *R. okamurae*, respectively, but this can be explained by species size differences. Density peaks of individuals were recorded in late summer and autumn, while biomass peaks were

observed in late spring and summer. This pattern suggests a seasonal variation in the species' invasiveness in *P. oceanica* meadows, with space occupation influenced either by the high influx of new recruits or the substantial biomass of larger adult individuals. A similar pattern in temporal fluctuation of invasive behaviour was observed in different invasive seaweeds, such as *S. muticum*, showing a population dynamic characterised by alternating periods dominated by either few large individuals or numerous small individuals (Arenas and Fernández 2000).

The lack of significant correlation between abiotic factors, density of individuals and biomass is useful for understanding the success and establishment of invasive species (Jehangir et al. 2024). This result suggests that *R. okamurae* can thrive under diverse physical-chemical conditions, tolerating a wide range of abiotic environmental factors, which enhances invasiveness (Alpert et al. 2000). A similar pattern was documented in *A. taxiformis*, which maintains a high productive succession of cohorts throughout the year, regardless of changing environmental conditions (Zanolla et al. 2019). During the study period, *R. okamurae* exhibited continuous recruitment, with peaks of young individuals observed in summer and autumn. Although the specific mechanisms involved were not detailed, the presence of propagules – diminute proliferous branches developed from cortical cells of the parent blade (Kajimura 1992) – was noticeable throughout the year, except in January (pers. obs.). This ability, coupled with its capacity for clonal multiplication through fragmentation (Rosas-Guerrero et al. 2020), facilitates ongoing recruitment regardless of abiotic factors. This mechanism not only adds to its continuous presence in the *P. oceanica* meadow, but also contributes to the release of new individuals into the water column. These unattached thalli can be detected at a depth of 1141 metres, where they remain photosynthetically active after light exposure (Mateo-Ramírez et al. 2023). This agrees with the propagule pressure invasion hypothesis, which emphasises the continuous input of new individuals into an invaded area (Simberloff 2009).

The identification of six successive cohorts throughout the year revealed the specific ecological strategies of *R. okamurae* for maintaining its population in the introduced area. The presence of different cohorts under varying environmental conditions suggests distinct environmental requirements for each cohort, a trait shared with other invasive species, such as *A. taxiformis* (Zanolla et al. 2019) and *U. pinnatifida* (Schiel and Thompson 2012). The population's prevalence is driven by constant recruitment and continuous generational succession. Most cohorts had a brief duration of about two months, except cohort IV, which lasted for six months. This finding suggests that the species is unable to complete the typical haplodiplontic digenetic life cycle exhibited in its native area, which usually spans more than two years and involves an annual alternation between gametophytic and sporophytic phases (Agatsuma et al. 2005). Although reproductive characterisation is still being processed, the continuous presence of propagules and monospores (pers. obs.) may indicate that the population is sustained primarily through asexual reproduction and vegetative multiplication, with sporophytes playing a key role in population maintenance. Furthermore, overlapping cohorts observed in some months allows for a continuous succession of individuals and sustained occupation of space resulting in high coverage throughout the year. This pattern, like that observed for *U. pinnatifida* in New Zealand, enhances the invasion success of the species and increases propagule pressure in invaded areas (Schiel and Thompson 2012; South et al. 2017). This continuous occupation can have more impact on the structure and function of the ecosystem than intermittent periods of

high and low coverage (Schiel and Thompson 2012) and is likely to disrupt native species and alter ecosystem dynamics, contributing to its invasive potential.

The identified cohorts might be linked to the occurrence of the species' morphotypes described in both native and introduced areas, characterised by variations in thallus thickness and width and the number of dichotomies (Sun et al. 2006; Salido and Altamirano 2020). These morphotypes appear to follow a seasonal pattern. For example, in introduced areas, the thick morphotype arises in winter and the thinner one in summer, alternating with the intermediate morphotype (Salido and Altamirano 2020). Therefore, cohort IV could align with the thicker morphotype, cohorts II and VI with the thinner morphotype and cohorts I, III and V with the intermediate morphotype. This potential association between morphotypes and cohorts provides valuable insights into the adaptability of the different morphotypes of *R. okamurae* in response to changing environmental conditions, which contributes to understanding the species' dynamics in marine ecosystems. Although this study did not explore the relationship between morphotypes and abiotic factors, identifying the environmental drivers behind morphotype development would be essential for understanding the species' invasion success and its impact on ecosystems. Theoretical frameworks exploring the connection between phenotypic plasticity and invasion success propose that invasive species could maintain high fitness across diverse environmental conditions owing to their physiological plasticity and can even thrive under unfavourable conditions (Richards et al. 2006; Gioria et al. 2023), which may explain the observed patterns in *R. okamurae*. Understanding this behaviour could deepen knowledge about the species' invasiveness and the factors influencing morphotype succession throughout the year.

Conclusion

Overall, *R. okamurae* exhibited a notable ability to persist in the *P. oceanica* meadow despite fluctuating environmental conditions. Its capacity to endure under a wide range of abiotic factors highlights its invasiveness, which is facilitated through population dynamics. This invasiveness observed in this specific habitat is further enhanced by continuous recruitment and a succession of distinct cohorts. This study also reveals temporal windows of invasiveness for *R. okamurae*, driven by an intense density of new small-sized individuals during summer and early autumn, while late spring and summer are characterised by high biomass accumulation of larger-sized individuals. These findings contribute to a deeper understanding of the invasiveness of *R. okamurae* in the *P. oceanica* meadows through population dynamics, underscoring its ability to dominate space and persist in a variety of environmental conditions. Identifying these patterns offers crucial insights into the success of *R. okamurae* and can guide effective management strategies.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: JRG, JDR, RC, MA. Data curation: JRG. Formal analysis: RC, MZ, JRG. Funding acquisition: JDR, RC, MA. Investigation: MA, RC, JRG, JDR, MZ. Methodology: JRG, MZ, JDR, MA, RC. Project administration: MA. Resources: MA. Supervision: MA. Validation: MA. Visualization: JRG. Writing – original draft: JRG. Writing – review and editing: JDR, RC, MZ, JRG, MA.

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Data availability

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

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Appendix 1

Table A1. One-way (month) ANOVA for density (individuals $\times 10^3$ per square metre) of *R. okamurae* and biomass (g DW per square metre). Asterisks represent significant differences ($P < 0.05$).

	variable	df	MS	F-value	P value
One-way ANOVA	Density	6	3761825	6.49	0.002*
	Biomass	6	9291	60.61	< 0.001*

Appendix 2

Table A2. Loading factors and percentage of the variance of the two first PCA.

	PC 1	PC 2
Percentage of variance	53.2%	26.6%
Cumulative percentage	53.2%	79.8%
Variable	PC 1	PC 2
Loading factors		
T	0.51	-0.05
Max T	0.49	0.10
Min T	0.49	-0.14
S	-0.30	0.01
LH	0.41	0.05
NO ₃	-0.01	0.70
NH ₄	0.04	0.69

T, average temperature; Max T, maximum average temperature; Min T, minimum average temperature; S, salinity; LH, Photoperiod, NO₃⁻, concentration of nitrate; NH₄⁺, concentration of ammonium.





Appendix 3

Table A3. Correlation analysis between two principal components and biological variables measured (density and biomass) in the population of *R. okamurae* (n = 7).

	PC 1		PC 2	
Variable	r	P value	r	P value
Density	0.57	0.18	0.24	0.61
Biomass	0.39	0.36	0.09	0.85

Research Article

Application of a theoretical simulator to the optimisation of risk-based invasive species surveillance

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Abstract

Early detection and rapid response are critical to the successful management of non-indigenous species (NIS) and rely on effective surveillance programmes. Risk-based surveillance, where surveillance targets high risk locations, is the most efficient form of NIS surveillance. However, further research is required on the impact of different levels of emphasis on risk, in sampling designs and on surveillance efficacy. This study implements a theoretical surveillance simulator to model the relative merit of different surveillance strategies with different levels of focus on NIS risk for NIS detection at one or more sites. Three potential surveillance scenarios were modelled: random, risk-based and heavy risk-based surveillance, each with three distributions of combined NIS risks of introduction and establishment: exponential, random and uniform. An example analysis using model derived NIS risk data is also provided. Sensitivity and elasticity analyses were conducted to identify variables which influence model outputs. The interaction between sampling method detection probability and changes in NIS abundance was modelled. It was found that NIS risk distribution influences the relative performance of different surveillance strategies and that risk- and heavy risk-based surveillance have lower times to detections and, generally, higher surveillance probabilities of detection compared to random surveillance at more skewed NIS risk distributions. However, there was a trade-off between short detection time and detection failure in risk-based and particularly heavy risk-based surveillance. Therefore, an over-emphasis on risk-based surveillance could provide suboptimal NIS detection. Sensitivity and elasticity analysis showed that the number of NIS seed sites, mean site visit rate and method detection probability had the largest effects on detection time, highlighting the complexity of designing surveillance programmes. In conclusion, the optimal surveillance strategy is conditional on the risk distribution and this study highlights the value of model-based simulators to guide decision-making in the design of NIS surveillance programmes.

Key words: Establishment risk, introduction risk, non-indigenous species, risk-based surveillance, surveillance design, theoretical model



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Introduction

Non-indigenous species (**NIS**) are species which have spread to new regions outside their natural biogeographical range with the aid of human actions (Essl et al. 2018). Some NIS can have substantial environmental impacts and are referred to as invasive NIS: they are the second largest driver of species extinction (Bellard et al. 2016), can reduce species genetic diversity (Vera-Escalona et al. 2019) and may have substantial ecological impacts (Gallardo et al. 2016; Guy-Haim et al. 2018), resulting in negative effects on ecosystem services (Vilà et al. 2010; Castro-Díez et al. 2019). They may also pose a direct threat to human health (Mazza et al. 2014) and have cost the global economy a minimum of 1.288 trillion US dollars from 1970 to 2017. This cost is rising every year (Diagne et al. 2021).

NIS introductions occur via various pathways. Five key pathways in the marine environment are commercial shipping, recreational boating, movement of aquaculture stock, the aquarium trade and natural dispersal (Ricciardi and Rasmussen 1998; Molnar et al. 2008; Acosta and Forrest 2009; Katsanevakis et al. 2013). Given multiple introduction pathways, the interception of all potentially invasive NIS is unrealistic. Prevention, early detection and rapid response are therefore critical to the successful management of NIS (Pyšek and Richardson 2010; Koch et al. 2020). This is particularly the case in the marine environment, where high environmental connectivity, via the water column, makes containment and eradication very challenging (Giakoumi et al. 2019). However, early detection can be difficult given that, when NIS arrive and become established, they are often initially rare (Hulme 2006; Koch et al. 2020). Early detection and rapid response rely on effective surveillance programmes. However, despite multiple legislative drivers (Wood et al. 2024), most European countries lack dedicated marine NIS surveillance programmes and NIS often receive limited attention in existing biological monitoring initiatives (Wood et al. 2021; Stæhr et al. 2022; Katsanevakis et al. 2023). Within the UK, the limited dedicated monitoring which does occur at marinas, remains spatially, temporally and taxonomically patchy (Bishop et al. 2015; Wood et al. 2017). In fact, the detection of NIS often occurs incidentally in more general environmental monitoring surveys (e.g. the detection of Pink Salmon, *Oncorhynchus gorbuscha*, in the Tees Estuary in northern England, UK, Gibson et al. (2024)).

Early detection and rapid response rely on effective surveillance programmes which must be in place prior to NIS arrival to allow early detection (Mastin et al. 2020). Risk-based surveillance, where surveillance targets high risk locations (e.g. where introduction pathway activity is high, Stärk et al. (2006); Tidbury et al. (2016)), is the most cost-effective form of hazard surveillance (Mastin et al. 2020; Stæhr et al. 2022). Risk-based surveillance may incorporate variation in establishment risk as well as introduction risk, given that the establishment risk of NIS varies spatially, based on parameters such as habitat suitability (Leidenberger et al. 2015; Richgels et al. 2016; Zhang et al. 2019). Risk-based surveillance confers efficiency by narrowing the survey sampling frame (Koch et al. 2020) and its use has been recommended for the early detection of colonising aquatic NIS based on empirical data (Harvey et al. 2009). However, risk-based surveillance is not always applied in practice. For example, in Europe and the UK, there is currently limited sampling at high-risk locations, for example, ports, marinas and aquaculture sites

for marine NIS (Stæhr et al. 2022; Wood et al. 2024). Therefore, further work is required to develop and apply risk-based surveillance methods. Risk-based surveillance is, however, a broad term, with surveillance strategies taking many forms with respect to the sampling effort used to monitor high-risk sites, relative to low-risk sites (i.e. the level of risk focus). Therefore, studying the effect of different levels of risk focus during sampling on NIS detection is a useful exercise when designing surveillance programmes.

Computer simulations which compare the effect of using simulated risk-based surveillance designs to random and other surveillance designs, on parameters which are of importance to NIS surveillance, such as time to detection or detection probability, provide a useful method to address this knowledge gap. These models vary in their sophistication and have been used in research into invasive plant pathogens (Parnell et al. 2014; Martinetti and Soubeyrand 2019; Mastin et al. 2020). While more general network models can evaluate the likely level of success of regional NIS management using multilayer network analysis (Garrett 2021), these models may have substantial species-specific data requirements (Martinetti and Soubeyrand 2019; Mastin et al. 2020). There is, thus, a requirement for the continued development of simple models which can be used to provide information for early warning surveillance of unanticipated new arrivals, where detailed information, underpinning prediction of their likelihood of spreading, is lacking (Parnell et al. 2014). This situation may apply to newly-introduced marine species. In fact, a lack of data on occurrence and distribution is a limiting factor in the response to marine NIS in the UK (Wood et al. 2024).

This study develops and implements a theoretical model, referred to as a surveillance simulator, to assess the relative merit of different surveillance strategies, which differ in their level of risk focus, for NIS detection. Although developed for early warning monitoring of marine NIS, where the species is established at a relatively small number of sites, the simulator is generic and can be applied to any terrestrial or aquatic organisms while requiring a minimal amount of species-specific data. The simulator calculates the time to NIS detection across multiple simulations, following the introduction and establishment of a NIS at one or more sites. The survey probability of detection, over time, is also calculated across simulations. Differential risk of introduction and establishment between sites is incorporated. Surveillance is simulated under three potential scenarios: random surveillance, risk-based surveillance and heavy risk-based surveillance. For risk-based surveillance, the visit rate is increased by the relative risk of NIS introduction and establishment. For heavy risk-based surveillance, this relative increase is enhanced for the highest risk sites. The simulator also incorporates the interaction between the detection probability of a method and changes in the abundance of NIS. Sensitivity and elasticity analyses are performed to determine the effect of changes in selected parameters on time to NIS detection and the failure to detect NIS. Findings are discussed in the context of optimisation of surveillance for NIS and the operation of the model rather than providing detailed differences between theoretical scenarios. Application of the model is further illustrated using NIS risk scores for 10,249 sites, derived from model predictions based on empirical data, for three scenarios focused on: risk of introduction, risk of spread and risk of impact and representing three different risk distributions.

Methods

Simulator structure

The simulator was developed in the statistical software R v.4.1.2 (R Core Team 2021). The simulator has several core components: functions which establish the NIS risk at each site (from the introduction and establishment probability) and which calculate the site visit rate based on surveillance strategy, the surveillance simulator function (run separately for each strategy) and functions which implement optional sensitivity and elasticity analyses (Fig. 1). Additional supporting functions process outputs and create graphs. The user inputs the following parameters: introduction and establishment probability distribution, mean annual visit rate, number of survey sites, the method detection probability, a minimum and maximum detection probability for the method and the detection dynamic. The detection dynamic indicates if the method detection probability remains constant or changes with NIS abundance at a site. The user specifies starting abundance changes according to a growth model with user-controlled parameters. The user may also specify the number of seed sites (sites at which NIS are introduced and become established) and the way in which detection outputs from multiple sites are summarised. The surveillance time period (in years) and number of simulations to run are also set. Definitions for parameters and other terms are in Table 1.

User-specified parameters

The input parameters are controlled via the `config_sim.yaml` file. The user specifies the number of sites and a probability of introduction (`getIntroProbability`) and establishment per site (`getEstablishProbability`). The distributions from which to randomly draw probabilities of introduction and establishment are either: an equal uniform distribution which requires a user specified probability value, random uniform distribution, truncated normal distribution (bounded by 0 and 1), truncated exponential (bounded by 0 and 1) or lognormal distribution (bounded by 0 and 1). Example distributions, used in the later simulator application example, are shown in Fig. 2. An overall NIS risk probability per site (Nr_s) is calculated: $Nr_s = Pi_s \cdot Pe_s$, where Pi_s is the probability of introduction per site and Pe_s is the probability of establishment per site.

A mean site visit rate is defined by the user and used to calculate the visit rate for each individual site. Under random surveillance, the visit rate for each individual site is identical. Under risk-based surveillance, the risk-based visit rate for each site (Vr_s) is calculated as: $Vr_s = V_s \cdot (Nr_s / Nr_{\bar{s}})$, where V_s is the visit rate per site and $Nr_{\bar{s}}$ is the overall mean NIS risk probability across sites. Under heavy risk-based surveillance, the visit rate for each site (Vhr_s) is calculated in the same manner, but the site NIS risk and mean NIS risk across all sites are raised to the power of three: $Vhr_s = V_s \cdot (Nr_s^3 / Nr_{\bar{s}}^3)$. Therefore, under the risk-based surveillance scenarios, the simulator assigns a relatively higher visit rate to those sites with greater NIS risk. Higher visit rates at high-risk sites are further enhanced under heavy risk-based surveillance. See Fig. 3 for a conceptual example of the relationship between NIS risk and site visits under different surveillance scenarios. If NIS abundance is included in the simulation, for a single site, a user-defined starting abundance value is set. For multiple

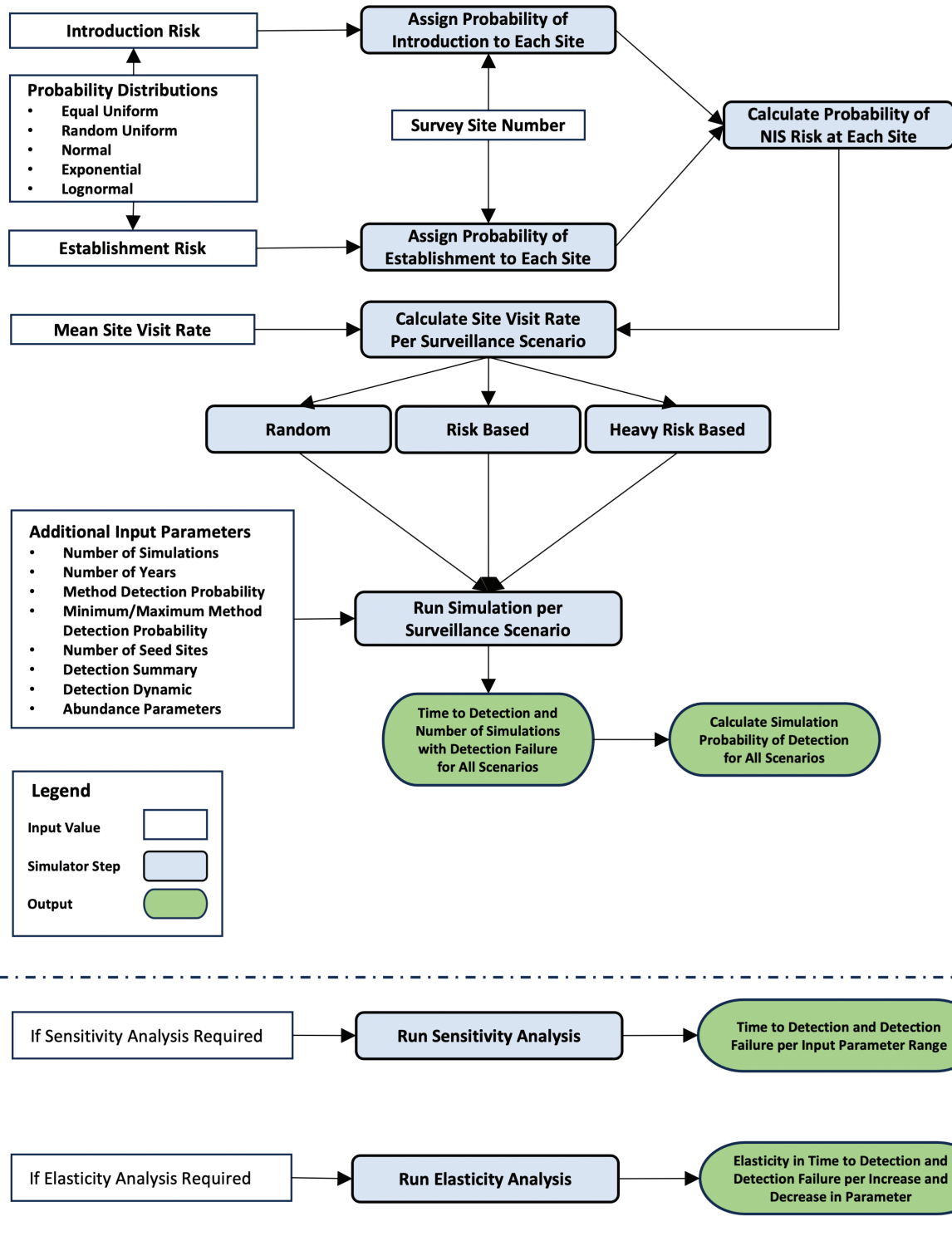


Figure 1. Schematic of the overall simulator structure showing key inputs and outputs and the role of the elasticity and sensitivity analysis, around the core surveillance simulation. The detailed structure of the surveillance simulations is given in Fig. 4.

sites, abundance starting values are set by the user or randomly drawn from a Poisson distribution with a user-specified mean. Abundance at each time step is determined by an exponential (Suppl. material 1: eqn. 1) or logistic growth model (Suppl. material 1: eqn. 2; Rockwood and Witt (2015)) with user-defined parameters (GetAbundance). This allows populations at site(s) to grow, decline or maintain at carrying capacity throughout the simulation.

Table 1. Glossary of key terms.

Parameter/ Term	Description*
Risk-Based Surveillance	Surveillance strategy where the site visit rate is biased towards higher risk sites.
Heavy Risk-Based Surveillance	Surveillance strategy where the site visit rate is heavily biased towards higher risk sites.
Introduction risk probability distribution	The statistical distribution which determines the probability of NIS introduction at a site.
Establishment risk probability distribution	The statistical distribution which determines the probability of NIS establishment at a site.
NIS risk	The probability of NIS introduction and establishment at a site, calculated by multiplying the introduction and establishment probability together.
Surveillance time period	The maximum time period (in years) over which a simulation may run.
Seed site(s)	A site(s) into which a NIS becomes introduced and established based on its relative NIS risk during a simulation.
Mean site visit rate	The mean number of times which a site is visited in a year.
Method detection probability	The probability of detecting a NIS at a site when it is searched during a simulation.
Survey probability of detection	The probability of detecting NIS at a site(s), at a given time point by a simulation, as calculated using all simulations in a simulator run.
Detection dynamic	The relationship between method detection probability and the abundance of a NIS. Either fixed, threshold or linear.
Detection summary	Method used to summarise the time to detection if multiple seed sites are used in a simulation.

*For further details, refer to text.

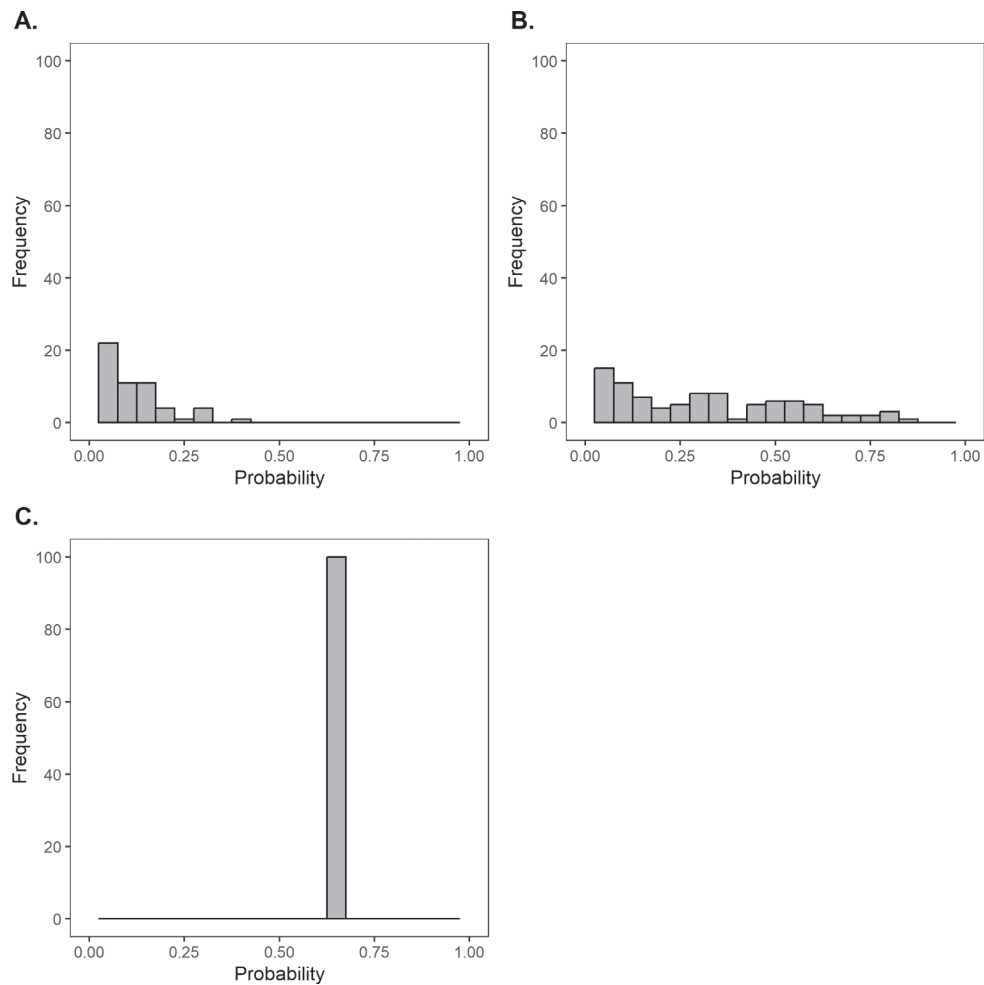


Figure 2. NIS risk distributions of the probability of NIS becoming introduced and established at a site, showing exponential (A), random uniform (B) and equal uniform (C) risk distributions used in the simulator application example.

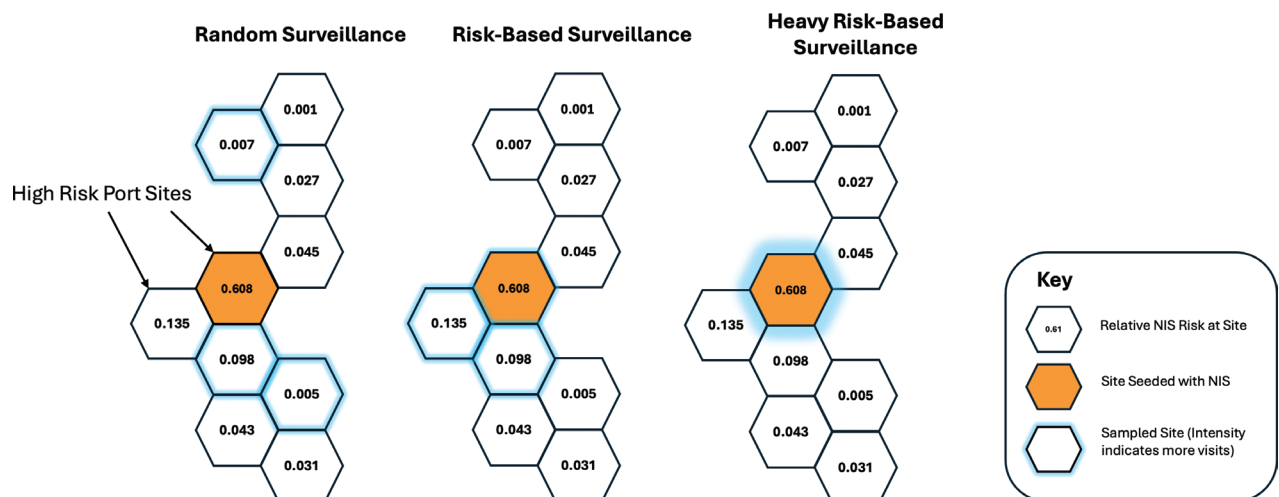


Figure 3. A conceptual example of the relationship between the relative NIS risk at each site (numbers within hexagons, assuming an exponential distribution) and the site visit rate assuming random, risk-based and heavy risk-based surveillance, over three site visits (blue outline) during a model run. The highest risk hexagons in this example represent two port sites, one seeded with a NIS at the beginning of the simulation (orange fill). Under random surveillance three sites are visited with no relationship to risk, under risk-based, three high risk sites are visited and under heavy risk-based surveillance, the highest risk site, only, is visited three times.

The method detection probability defines the probability of the sampling method detecting the NIS during a site visit. The method detection probability may be fixed or vary with NIS abundance linearly or in a threshold manner. Under a linear relationship, the user defines the abundance required to change the detection probability by 0.01. Under a threshold relationship, the user defines a threshold abundance value and two detection probabilities to use when abundance is below, above or equal to the threshold value.

Introductions at multiple seed sites, up to the number of sites in the simulation, may also be selected by the user. If abundance is required, values for multiple sites are either set by the user or randomly drawn from a Poisson distribution with a user specified mean. For multiple sites, the user must select the detection summary method, i.e. how the time to detection is summarised over multiple seed sites in that simulation (ProcessMultipleResults). Time to detection may be taken from the first seed site to be detected or the last seed site.

Simulation process

The simulation is run by the function runSurveillanceSimulation (Fig. 4). At the starting time point, a site is selected with its relative NIS risk used as a probability weighting to bias random selection to higher NIS risk sites (base R function: sample) and seeded with a NIS (Fig. 3). For simulations which include multiple (n) seed sites, this process is repeated n times (once for each seed site). The simulator time step (in days) is calculated by dividing the user-defined number of visits per year by 365, assuming that the total visits each year is equal to the sum of visit rates across all sites. The time counter is increased, based on the average time taken to visit one site assuming that the total visits each year is equal to the sum of visit rates across all sites.

At each time step, a single site is selected to be visited dependent on the mean visit rate (Fig. 3). At each site visit, detection of the NIS is determined by drawing a value from a random binomial distribution with a success rate

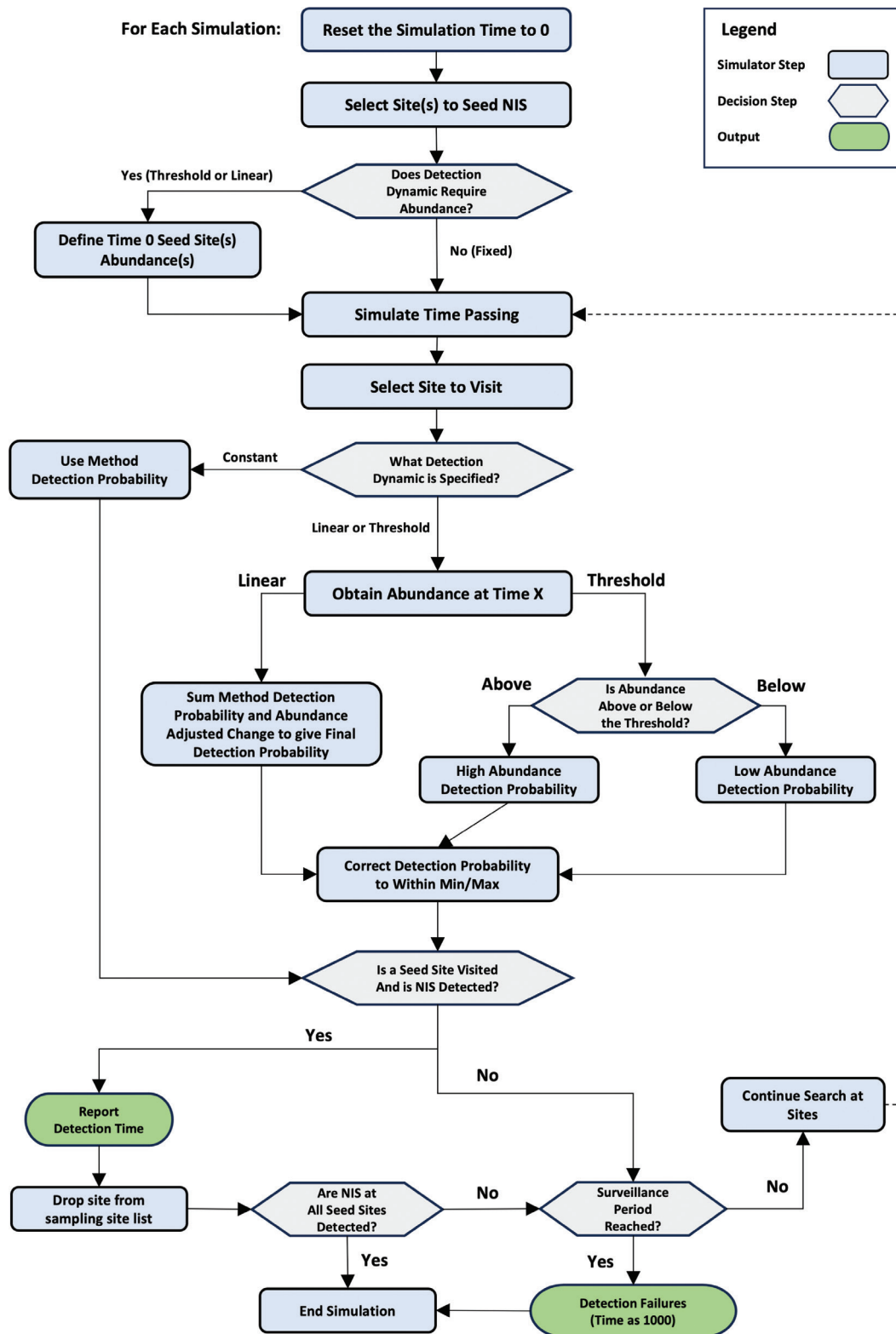


Figure 4. Schematic of the surveillance simulation showing key steps and outputs, as defined by the *runSurveillanceSimulation* function.

defined by the method detection probability. If the seed site is visited and the NIS is successfully detected, the simulation stops and the time to detection is recorded. When NIS are seeded at multiple sites and when NIS is detected at a seeded site, the time is stored and the simulation continues until NIS is found

at all sites. The multi-site simulation will run until NIS are detected at all seeded sites or the surveillance period elapses. If the simulation period elapses prior to the NIS being detected, the run is stored as a 'detection failure' (and internally stored as time to detection = 1000). The results for multiple seed sites are then summarised by the function `ProcessMultipleResults`, for each simulation. The default detection summary method, used in this study, is used to output the time at which the last seed site was detected. This means that the results of multiple seed sites are counted as a detection failure when NIS remain undetected at even just one of the seeded sites within the timeframe. Other detection summary options can output the mean, median or first time to detection across seed sites or the time taken to detect a user-specified number of sites. The simulator repeats according to the number of simulations set. The outputs are time to detection and proportion of total simulations which are classed as 'detection failures' (across all simulations). This second output is also used to calculate the survey probability of detection over all sites across time. Outputs across all surveillance simulations are generated by the `report-NIS-intro-detect-sim.Rmd` markdown file.

Sensitivity and elasticity analysis

Sensitivity analysis determines the impact that absolute changes in each model parameter have on the output, i.e. time taken to detect NIS and forms a component of the surveillance simulator. Sensitivity analysis can be implemented for the number of sites, number of years, mean visit rate, method detection probability and number of seed sites (`makeSensitivityParamsTable`). The simulator runs iteratively (`runSurveillanceSensitivity`; results formatted by `formatSensitivityResults`), incrementally altering input parameters, one at a time, by a user-defined interval within a specified range and plotting the results. Summary statistics such as number of times a NIS was detected/not detected and the mean, maximum and minimum time to detection are output for each parameter. Outputs are generated by the `report-NIS-intro-detect-sensitivity.Rmd` R Markdown file and other helper functions.

Elasticity analysis is also included in the simulator. Elasticity (ξ) is proportional sensitivity, it estimates the effect of a proportional change in a parameter on the proportional change in the output, i.e. time taken to detect NIS (Benton and Grant 1999; Teixeira Alves et al. 2021). Elasticity is dimensionless and independent of the parameter scale, allowing comparison between parameters. Elasticity analysis can be implemented and compared with sensitivity analysis to better understand the impact of changes in model parameters on outputs. Users define the default parameter values and the proportion (between 0 to 1) by which to change each parameter (defined in the `config_sim.yaml` file; `makeElasticityParamsTable`). The simulator is run iteratively, with one parameter varied at a time (using `runSurveillanceSensitivity`; results formatted by `summariseElasticityResults`). Elasticity is calculated (Suppl. material 1: eqn. 3; Teixeira Alves et al. (2021)) and plotted for each parameter (by `report-NIS-intro-detect-elasticity.Rmd` and other helper functions). Elasticity values below 1 indicate a parameter is inelastic. Elasticity values above 1 indicate the parameter is elastic, i.e. changes in elastic parameters have the greatest impact on outputs (Teixeira Alves et al. 2021).

Simulator application

Theoretical risk distributions

Three different NIS introduction and establishment risk distributions were implemented for each surveillance strategy (random, risk-based and heavy risk-based). These risk distributions were equal uniform (probability: 0.8), random uniform and exponential. The equal uniform distribution was selected to provide a default example with no variation in risk. The random uniform distribution provided a scenario where risk varied between sites, whereas the exponential distribution was used to represent a situation where most sites are of no or low risk and a small number of sites are of high risk (Wood et al. 2021). All other parameters were kept constant between runs. The number of seed sites was 1. This was used for baseline comparisons because the aim of the simulator is to optimise detection of NIS early after arrival. The number of years was set to 30, to allow the majority of simulations to detect the NIS and, therefore, provide valid comparisons between detection times. All other parameters were selected according to the authors' knowledge of sampling programmes. Specifically, the number of survey sites was 100, mean visit rate was 1 and method detection probability was 0.8. The number of simulations in each run was set to 10,000 as experimentation showed that simulator outputs were consistent between identical runs at this number of simulations.

Sensitivity and elasticity analysis

For sensitivity and elasticity analysis, the exponential risk distribution was used as, under this risk distribution, the largest differences between sampling programmes were seen. For the sensitivity analysis, the number of seed sites, survey sites, years, mean visit rate and detection probability were run with selected parameters defined, based on the authors' knowledge of sampling programmes (Table 2). For the elasticity analysis, the default parameters $\pm 25\%$ for the number of sites, number of years, mean visit rate and method detection probability from the sensitivity analysis were used (Table 2), as they were considered practically sensible and allowed clear comparison between parameters. The number of seed sites was not included in the elasticity analysis as it was difficult to generate proportional increases in the default number of seed sites (i.e. 1 seed site), which would have an impact on the simulations.

Detection dynamic

The effect of dynamic detection (where the method detection probability is linked to NIS abundance) was explored using an exponential risk distribution and with seed site set to 1 and 10. The abundance model parameters assumed a starting population of 1, intrinsic growth rate of 1.5 with logistic growth and a population carrying capacity at each site of 100,000 individuals. For a linear relationship between abundance and detection method sensitivity, the starting detection method sensitivity was set to 0.1 with an increase of 0.01 per abundance increase of 500, up to 0.8. For a threshold relationship between abundance and detection method sensitivity, an abundance threshold of 10,000 was set such that method detection sensitivity below and above this threshold was 0.1 and 0.8, respectively. Parameter values were arbitrarily selected to demonstrate the functionality of the simulator.

Table 2. Sensitivity and elasticity parameters.

Parameters	Sensitivity Values			Elasticity Values		
	Minimum	Maximum	Interval	Default	25% Decrease	25% Increase
Number of Seed Sites	1	100	10			
Number of Survey Sites	50	200	25	100	75	125
Number of Years	10	50	5	30	22.5	37.5
Mean Visit Rate	0.25	4	0.25	1	0.75	1.25
Method Detection Probability	0.1	1	0.1	0.8	0.6	1.0

‘Site Prioritisation Tool’ derived marine NIS risk distributions

As a practical example, the surveillance simulator was used to assess the relative performance of random, risk-based and heavy risk-based surveillance for three marine NIS introduction and spread scenarios created by a model to prioritise surveillance activities for NIS species for the UK coastline (the ‘Site Prioritisation Tool’ or SPT model, Cefas, in prep.). This hierarchical model was developed to provide information for surveillance programmes by scoring and ranking 10,249/5 km × 5 km grid squares representing the UK coastline. Empirical data for a range of risk parameters was grouped into pathways, distributed amongst four risk categories: Introduction risk (pathways: intentional introduction, shipping, recreational boating, fishery and aquaculture release), Establishment risk (temperature, salinity and substrate), Impact risk (environment and industry) and Spread risk (recreational boating, fishery and aquaculture release; Suppl. material 1: table S1). Separate weighting factors were assigned to each parameter, theme and category to reflect their relative importance and determine their contribution to risk scores. Resulting risk scores are standardised (between 0 and 1) at each level of the SPT model (parameter, pathway, category) to provide comparable relative values between pathways and categories. Risk scores were output for three scenarios: Scenario A, monitoring weighted towards sites at greatest risk of introduction through the shipping pathway (e.g. species introduction via ballast water and hull fouling); Scenario B, monitoring weighted towards sites where spread risk is greatest; and Scenario C, monitoring weighted towards sites where the impact of NIS is likely to be greatest (Suppl. material 1: fig. S1). The simulator was run with NIS risk scores from each scenario (Fig. 5), with the per cell risk data from the SPT model used to provide the overall NIS risk probability per site (Nrs). The simulator was run for 1000 simulations, at 10,249 sites (grid cells), to determine time to detection assuming a constant detection dynamic with all other parameters as default (as in section Theoretical risk distributions).

Results

The risk distribution

Comparison of the time to detection between different risk distributions showed that results varied with surveillance strategy (Table 3; Fig. 6). For an equal-uniform distribution, there was almost no variation in time to detection or survey probability of detection between surveillance strategies. In addition, all NIS were detected regardless of surveillance strategy (Table 3; Fig. 6C). With a random uniform risk distribution, the median time to detection was 0.89 years for random surveillance

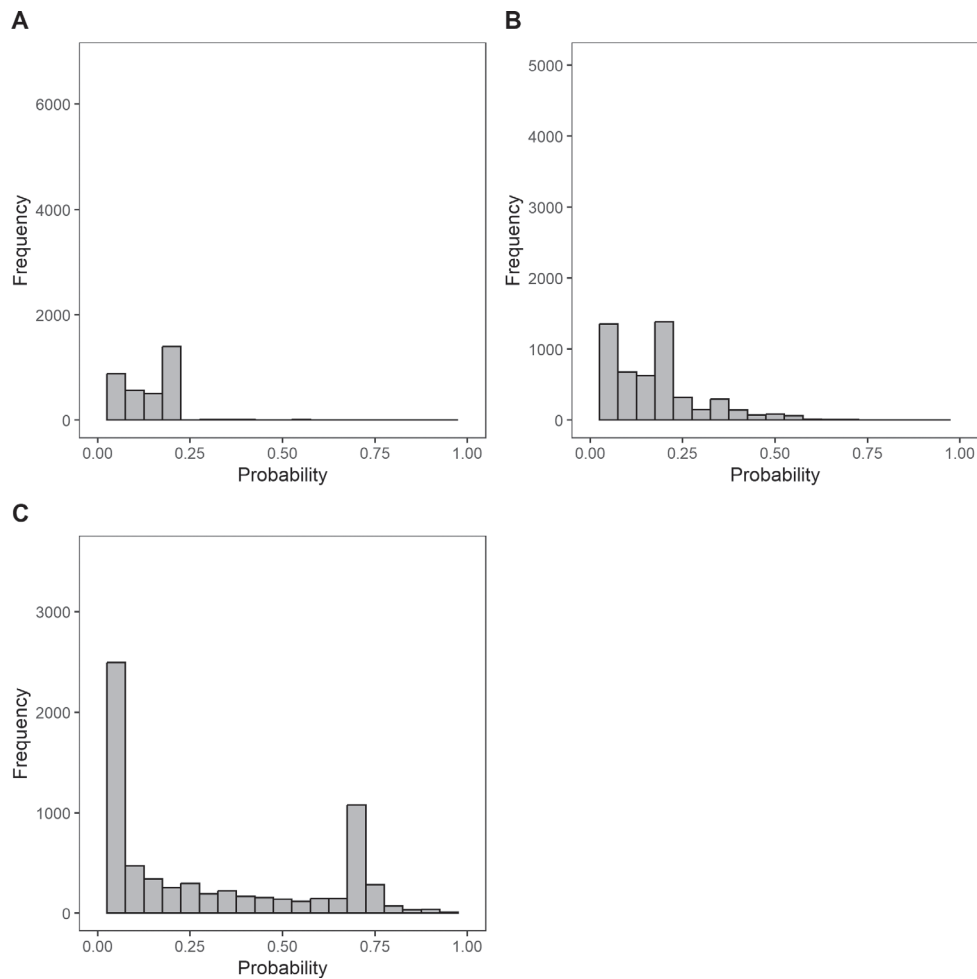


Figure 5. Risk distributions of the combined probability of marine NIS becoming introduced and established at a site, spread from that site and the site being negatively impacted. Scores generated using the SPT model (Cefas, in prep.) for Scenario **A** shipping risk weighted, Scenario **B** spread risk weighted and Scenario **C** impact risk weighted.

and decreased to 0.56 years for risk-based surveillance and 0.52 years for heavy risk-based surveillance. Probability of detection at 1 year was highest for risk-based surveillance (0.68) and was progressively lower for heavy risk-based (0.61) and random surveillance (0.54). However, heavy risk-based surveillance had the lowest detection probability after 5 years (Table 3; Fig. 6B). Under an exponential risk distribution, median time to detection was longest under random surveillance (0.85 years) and was shortest under risk-based surveillance (0.41 years), but the time to detection under heavy risk-based surveillance was marginally longer than risk-based surveillance (0.47 years). Probability of detection at 1 year showed risk-based surveillance to have the highest survey probabilities of detection (0.75) and random and heavy risk-based surveillance to have similar lower scores (0.56 and 0.57). However, heavy risk-based surveillance had the lowest detection probability after 5 years (Table 3; Fig. 6A). For risk-based and heavy risk-based surveillance, time to detection progressively fell from an equal uniform, random uniform to an exponential risk distribution (Table 3). Risk-based surveillance showed a progressive increase in probability of detection, but heavy risk-based surveillance showed a limited change across distributions. For random surveillance, the time to detection and probability of detection remained the same across risk distributions and NIS were always detected (Fig. 6; Table 3). Under exponential and random uniform

Table 3. Model outputs for variable risk distributions.

Model Run	Distribution	Detection Dynamic	Number of Seed Sites	Scenario	Detection Time (Years)		Detection Failure (%)	Survey Probability of Detection at Time (Years)			
					Median Detection Time	Interquartile Range		1	5	10	30
Run 1	Exponential	Constant	1	Random	0.85	1.34	0.00	0.56	0.98	1.00	1.00
				Risk-Based	0.41	0.85	0.28	0.75	0.96	0.99	1.00
				Heavy Risk-Based	0.47	1.86	10.68	0.57	0.78	0.83	0.89
Run 2	Random Uniform		1	Random	0.89	1.39	0.00	0.54	0.98	1.00	1.00
				Risk-Based	0.56	1.01	0.15	0.68	0.97	0.99	1.00
				Heavy Risk-Based	0.52	1.45	7.49	0.61	0.84	0.88	0.93
Run 3	Equal Uniform		1	Random	0.87	1.39	0.00	0.54	0.98	1.00	1.00
				Risk-Based	0.86	1.36	0.00	0.56	0.98	1.00	1.00
				Heavy Risk-Based	0.88	1.35	0.00	0.55	0.98	1.00	1.00
Run 4	Exponential	Linear	1	Random	6.03	4.39	0.00	0.09	0.40	0.97	1.00
				Risk-Based	3.34	5.03	0.50	0.20	0.63	0.95	0.99
				Heavy Risk-Based	3.55	6.70	12.48	0.24	0.49	0.76	0.88
Run 5			10	Random	8.79	1.78	0.00	0.00	0.00	0.78	1.00
				Risk-Based	8.31	3.51	3.02	0.00	0.02	0.68	0.97
				Heavy Risk-Based	14.52	11.25	68.87	0.00	0.00	0.08	0.31
Run 6	Exponential	Threshold	1	Random	6.29	4.23	0.00	0.09	0.39	0.97	1.00
				Risk-Based	3.20	5.20	0.29	0.21	0.63	0.96	1.00
				Heavy Risk-Based	3.63	6.54	12.39	0.24	0.49	0.76	0.88
Run 7			10	Random	8.61	1.79	0.00	0.00	0.00	0.81	1.00
				Risk-Based	8.14	3.54	3.16	0.00	0.01	0.69	0.97
				Heavy Risk-Based	14.71	11.45	67.67	0.00	0.00	0.09	0.32

risk distributions, NIS are not always detected within 30 years using risk-based and heavy risk-based surveillance. Under a random uniform risk distribution, 0.15% of simulations ended with no NIS detected with risk-based surveillance and this increased to 7.5% under heavy risk-based surveillance (Table 3). For an exponential risk distribution, 0.28% of simulations ended with detection failure for risk-based surveillance and this increased to 10.68% of simulations for heavy risk-based surveillance (Table 3).

Sensitivity analysis

Assuming an exponential risk distribution, an increase in the number of seed sites from 1 to 20 led to an increase in median time to detection across surveillance scenarios: from 0.87 to 3.6 years for random surveillance, 0.35 to 4.11 years for risk-based surveillance and 0.43 to 17.61 years for heavy risk-based surveillance (Fig. 7A), although there was substantial variability in the results. At 30 seed sites or greater, time to detection decreased across surveillance scenarios from 3.69 years for random surveillance, 3.82 years for risk-based surveillance and 17.02 years for random surveillance to 1.25 years at 100 seed sites across all scenarios (Fig. 7A). This decrease was the consequence of the adaptive sampling design where sites were not revisited after NIS detection, thereby creating a smaller pool of sites from which to sample at each step, therefore reducing the time to detect all sites with a

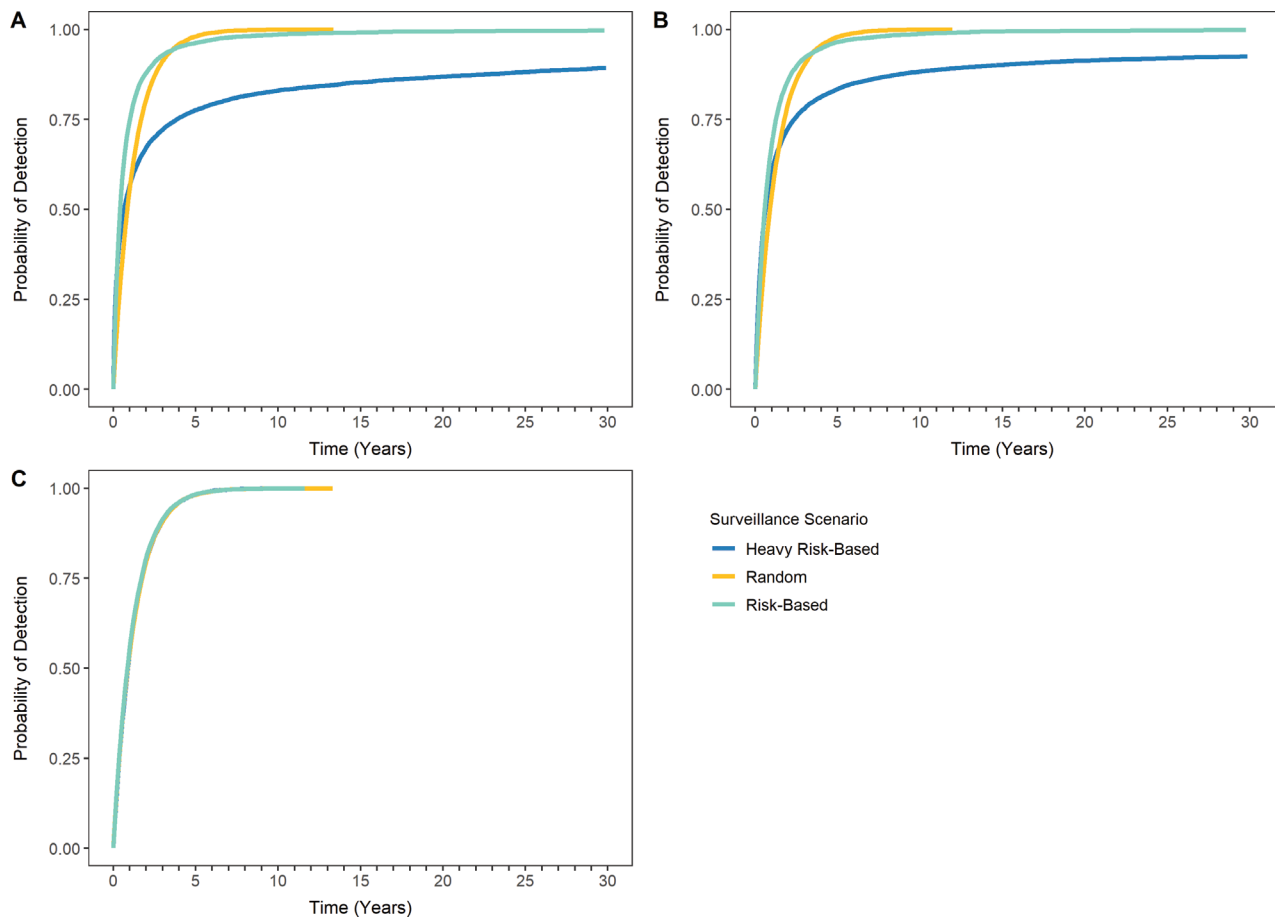


Figure 6. The overall survey detection probability of NIS over time, calculated across 10,000 simulations, assuming an exponential (A), random uniform (B) and equal uniform (C) risk distribution.

NIS. This effect was most pronounced where all 100 sites used in the simulation were seeded with NIS. Relative differences in median detection time showed heavy risk-based surveillance performed extremely poorly with more than one seed site, whereas random and risk-based scenarios both showed much lower and comparable median times to detection (Fig. 7A). The percentage of simulations from which the NIS was not detected was much higher for heavy risk-based surveillance compared to risk-based surveillance, but risk-based surveillance showed a similar relative trend to heavy risk-based surveillance in changes to detection failures with the number of seed sites (Fig. 7A). For example, with 20 seed sites, heavy risk-based surveillance failed to detect NIS in 89.2% of simulations, whereas this number was only 2.2% for risk-based surveillance.

The number of sampling sites had little impact on the median time to detection or the detection failure of random or risk-based surveillance (Fig. 7B). Heavy risk-based surveillance showed some small effect of number of sampling sites on median time to detection and detection failure (Fig. 7B). The number of sampling years had no influence on the median time to detection or detection failure of random surveillance (Fig. 7C). However, there was an increase in the number of simulations which were long time to detection outliers in both risk and heavy risk-based surveillance as the number of sampling years increased (Fig. 7C). Comparably, detection failure fell slightly across risk and heavy risk-based surveillance as the number of sampling years increased (Fig. 7C). Increases in mean visit rate, from

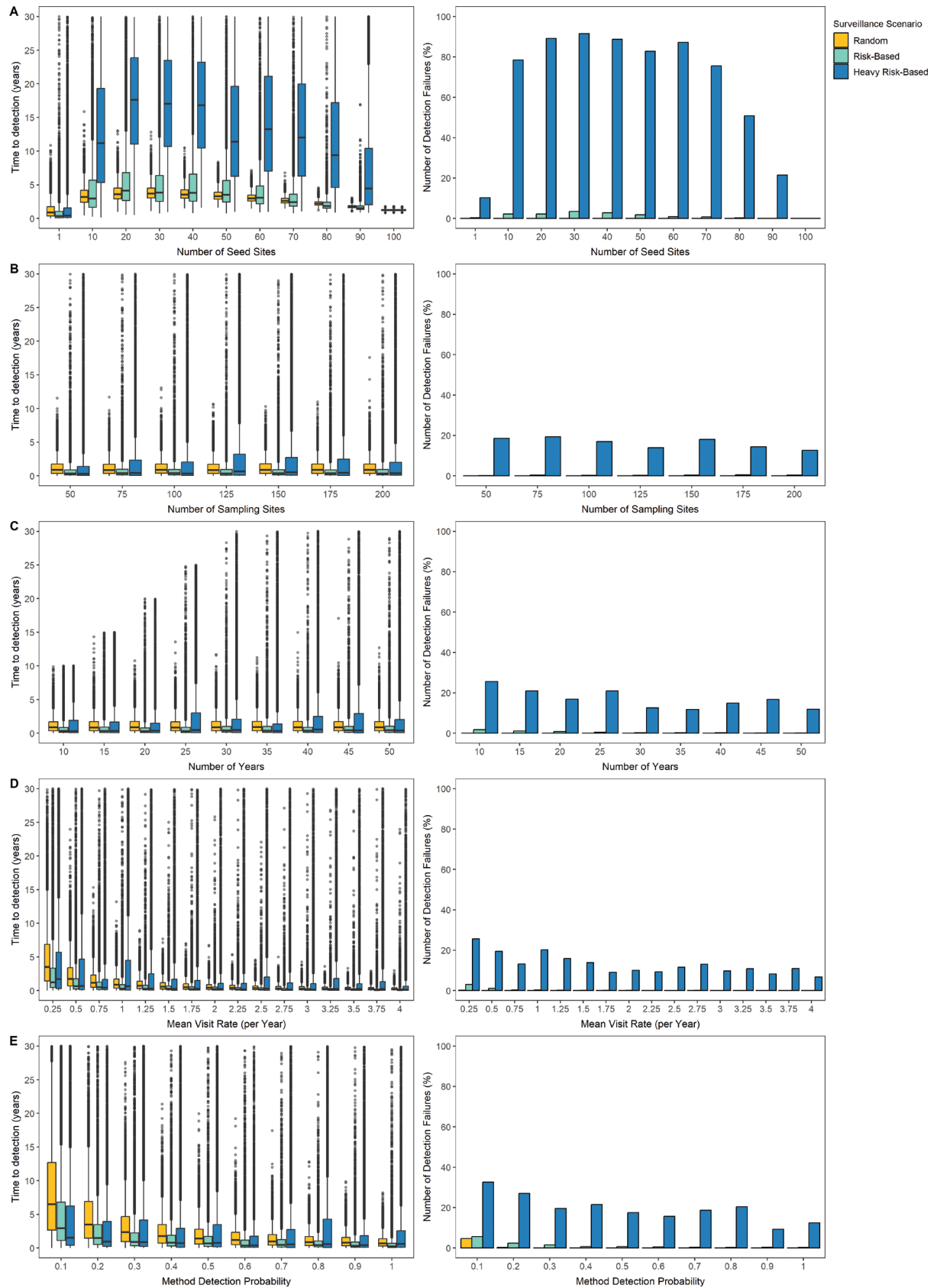


Figure 7. The results of the sensitivity analysis, assuming an exponential risk distribution, for the parameters: number of seed sites (A), number of sampling sites (B), number of sampling years (C), mean visit rate (D) and method detection probability (E), showing their effect on median time to detection (left hand column) and the percentage of simulations in each model run where no NIS was detected (right hand column).

0.25 to 1 visits per year, caused a decline in median time to detection across scenarios, for risk-based (from 1.25 to 0.30 years), heavy risk-based (1.68 to 0.64 years) and random surveillance (3.49 to 0.88 years, Fig. 7D). Time to detection continued to decline with increasing visit rates above one per year, though this was less pronounced for risk-based surveillance (Fig. 7D). There were overall declines in detection failure across surveillance scenarios as mean visit rate increased, although this relationship was non-linear (Fig. 7D). This was particularly evident for heavy risk-based surveillance (Fig. 7D). For random surveillance, an increase in method detection probability from 0.1 to 0.5 caused the median time to detection to fall from 6.49 to 1.40 years (Fig. 7E). Detection failure under random surveillance also fell from 4.7% at detection probability 0.1, to 0.0% at detection probability 0.3 (Fig. 7E). Under risk-based surveillance a similar, but more subtle, decline for median time to detection occurred over the same range (2.93 to 0.67 years, Fig. 7E). Detection failure also decreased, but never reached zero. Median time to detection under heavy risk-based surveillance showed little response to method detection probability between 0.1 to 0.5 (1.51 to 0.75 years, Fig. 7E). However, detection failure decreased overall as method detection probability increased (Fig. 7E).

Elasticity analysis

Assuming an exponential distribution, the elasticity of median time to detection and detection failure varied between surveillance scenarios, parameters and the direction of change in parameter values (Fig. 8). Under random surveillance, no change in detection failure was seen over the parameter ranges; therefore, elasticity was not calculated (Fig. 8B). Median time to detection was generally inelastic and only elastic to increases in the number of sites sampled under risk-based surveillance ($\xi = 1.07$, Fig. 8A). Under heavy risk-based surveillance, the detection failure was elastic to a decrease ($\xi = 2.32$) and increase ($\xi = 1.15$) in the number of sites sampled (Fig. 8B). Median time to detection was generally inelastic to changes in the number of years, but was elastic to an increase ($\xi = 1.05$) and decrease ($\xi = 1.20$) in the number of simulation years under risk-based and heavy risk-based surveillance, respectively (Fig. 8A). Detection failure generally showed an elastic response to the number of simulation years, though an increase in the number of years was inelastic under risk-based surveillance (Fig. 8B). Median time to detection generally showed an elastic response to mean visit rate, in particular under heavy risk-based surveillance, where time to detection showed strong elasticity to reductions in mean visit rate ($\xi = 2.21$, Fig. 8A). However, time to detection was inelastic to an increase in mean visit rate under random surveillance (Fig. 8A). Detection failure was elastic to increases in mean visit rate under risk-based surveillance ($\xi = 1.70$, Fig. 8B). Median time to detection was generally inelastic to changes in the method detection probability, except under risk-based surveillance where it was elastic to increases in the method detection probability ($\xi = 1.55$) and under random surveillance where it was elastic to decreases in the method detection probability ($\xi = 1.33$, Fig. 8A). Detection failure was generally elastic to changes in the method detection probability, particularly to a reduction in detection probability under risk-based surveillance ($\xi = 2.55$, Fig. 8B). Under heavy risk-based surveillance, detection failure was inelastic to an increase in method detection probability (Fig. 8B).

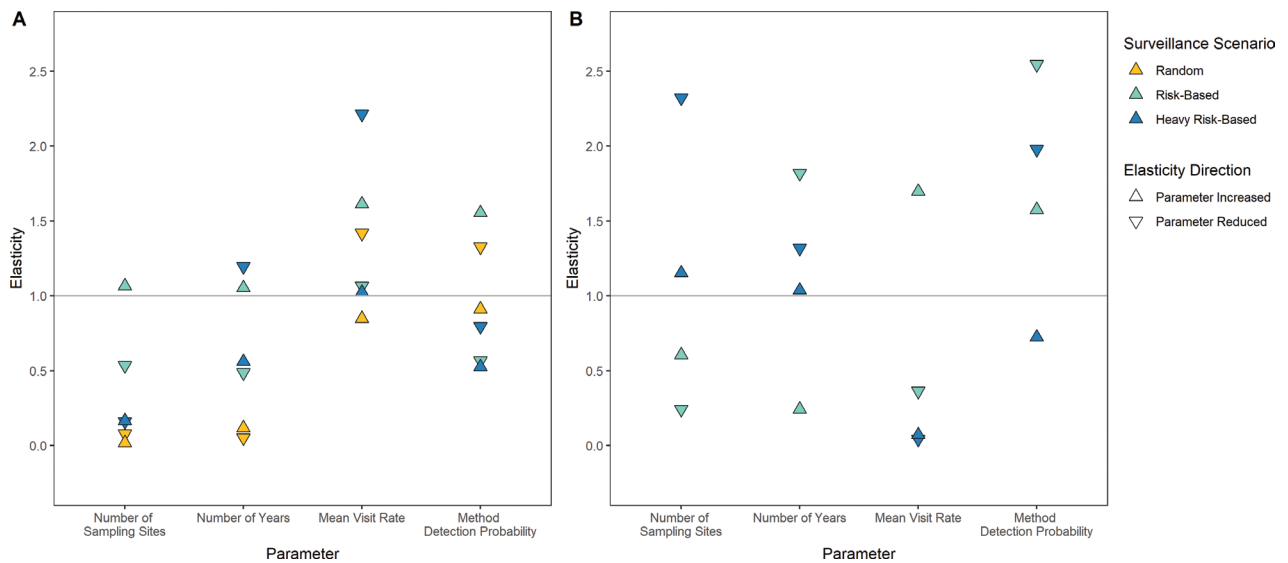


Figure 8. The elasticity of the median time to detection (years; **A**) and the detection failure (%; **B**) to a 25% increase and decrease in the default values of mean visit rate, number of sampling sites, number of years and method detection probability in each model run, assuming an exponential risk distribution.

Detection dynamic

Assuming an exponential risk distribution, inclusion of a linear relationship between method detection probability and NIS abundance (i.e. a linear detection dynamic), which grew logistically, resulted in a similar pattern of median time to detection and detection failure, between surveillance scenarios, for one seed site (model run 4; Table 3) compared to when the relationship with NIS abundance was excluded (model run 1; Table 3). However, median times to detection were much longer for all scenarios (random: 6.03 vs. 0.85 years, risk-based: 3.34 vs. 0.41 years and heavy risk-based surveillance: 3.55 vs. 0.47 years) when a linear relationship was included vs. excluded (Table 3). In addition, detection failure was marginally higher for risk-based (0.50 vs. 0.28%) and heavy risk-based surveillance (12.48 vs. 10.68%). Survey probability of detection had a different relationship when a linear detection dynamic was included: at 1 and 5 years, random surveillance had the lowest value, with detection probability being higher in risk- and heavy risk-based surveillance (Table 3, Fig. 9A). However, this changed over time, when, at 10 years, heavy risk-based surveillance had the lowest probability of detection (Table 3, Fig. 9A). When 10 seed sites were included with a linear detection dynamic, median time to detection lengthened for all surveillance scenarios (model run 5; Table 3). Risk-based surveillance had the shortest time to detection as before (8.31 years), but heavy risk-based surveillance had a much longer median time to detection (14.52 years) compared to random surveillance (8.79 years), in contrast to model runs 4 and 1 (Table 3). Heavy risk-based surveillance had a higher detection failure (68.87%) compared to risk-based surveillance (3.02%, Table 3). Survey probability of detection was at or near zero for all scenarios at 1 and 5 years. It remained low for heavy risk-based surveillance at 10 and 30 years, but increased for random and risk-based surveillance, with random surveillance having the highest value from 10 years (Table 3, Fig. 10B).

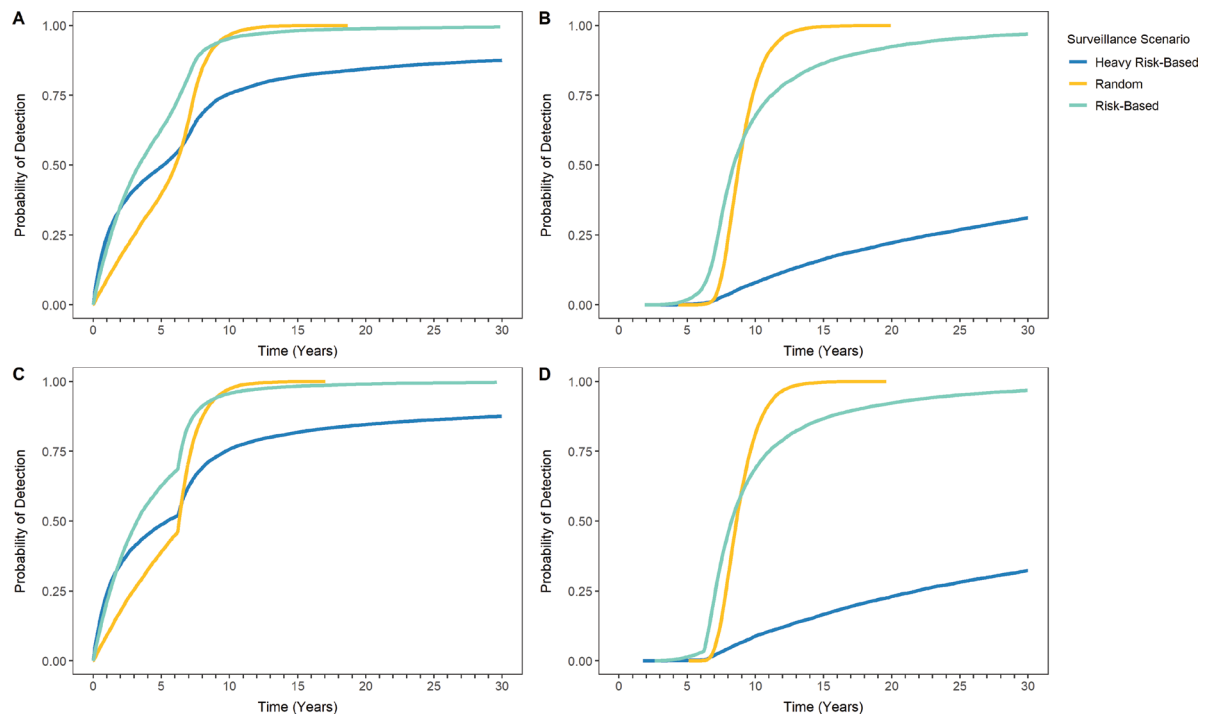


Figure 9. The survey probability of detection of NIS at a site, or all sites, over time, assuming an exponential NIS risk distribution, calculated across 10,000 simulations, for a linear (panels **A** and **B**) and threshold detection dynamic (panels **C** and **D**) between NIS abundance and method detection probability for one (left hand panels) and ten seed sites (right hand panels).

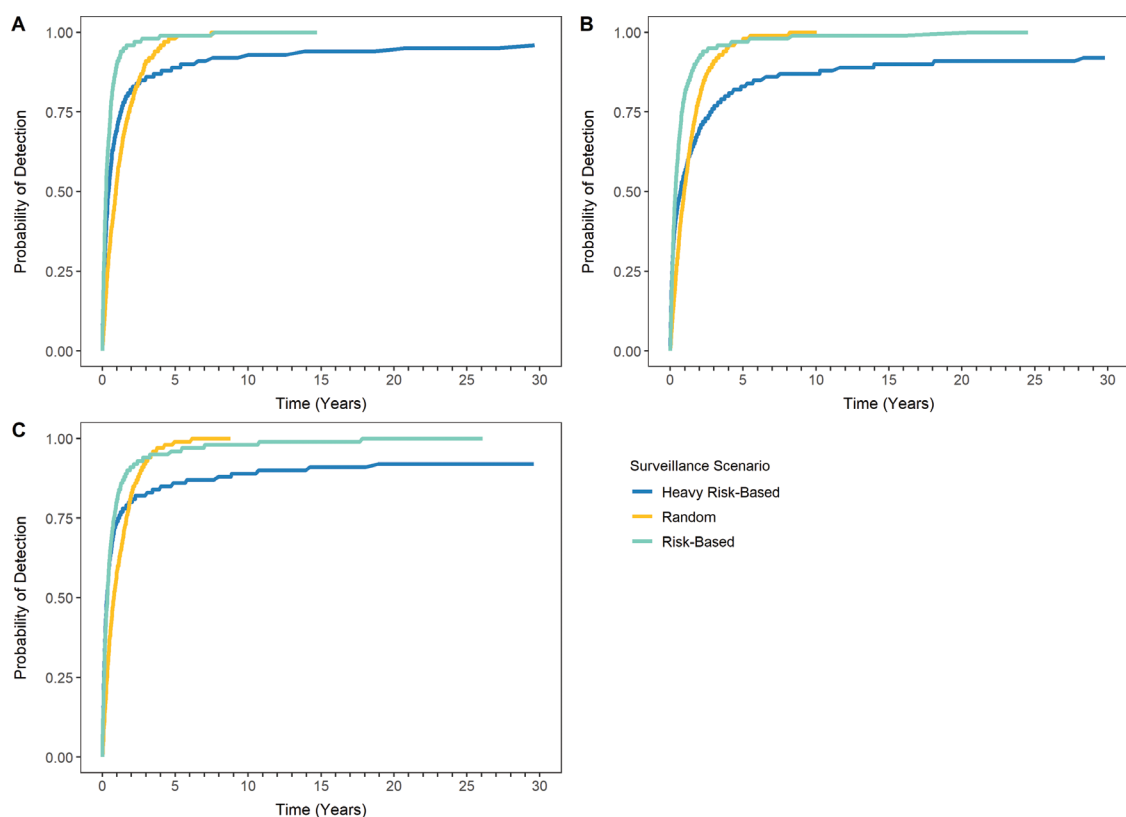


Figure 10. The overall survey detection probability of NIS over time, calculated across 1000 simulations, assuming the risk distribution in the combined probability of NIS becoming introduced and established at a site, spread from that site and the site being negatively impacted. Scores generated using the ‘Site Prioritisation Tool’ (Cefas, in prep.) for Scenario **A** shipping risk weighted, Scenario **B** spread risk weighted and Scenario **C** impact risk weighted.

Assuming a threshold detection dynamic between method detection probability and NIS abundance, simulations with one seed site (model run 6) produced similar median times to detection and detection failure to a linear detection dynamic with one site (model run 4), across surveillance scenarios (Table 3). Similarly, when 10 seed sites (model run 7) were included using a threshold detection dynamic, the results were similar to those with a linear detection dynamic with 10 seed sites (model run 5), in that heavy risk-based surveillance had the longest time to detection (14.71 years) and highest detection failure (67.67%, Fig. 9; Table 3). Detection probabilities showed a similar pattern across scenarios and simulations to those for a linear detection dynamic (Fig. 9C, D).

‘Site Prioritisation Tool’ derived marine NIS risk distributions

The time to detection and other outputs varied between surveillance strategies for each SPT modelled site risk distribution (Table 4; Fig. 10). For Scenario A (shipping risk weighted), the median time to detection was shortest for risk-based surveillance (0.25 years) and progressively longer for heavy risk-based (0.37 years) and random surveillance (0.93 years, Table 4). The survey probability of detection at Year 1 was also highest for risk-based surveillance (0.90) compared to heavy risk-based (0.70) and random (0.53) surveillance (Table 4, Fig. 10A). Heavy risk-based surveillance, however, had the highest detection failure (4.40%) compared to risk-based and random surveillance (both 0.00%, Table 4). Scenario B (spread risk weighted) had overall similar relative results to Scenario A: risk-based surveillance had the shortest detection time (0.34 years) and highest survey probability of detection at Year 1 (0.81, Table 4, Fig. 10B). However, risk-based surveillance also showed detection failure (0.30%), whereas random surveillance did not (0.0%, Table 4). For Scenario C (impact risk weighted), median time to detection was shortest for heavy risk-based surveillance (0.27 years), compared to risk-based (0.34 years) and random surveillance (0.78 years, Table 4). Similar to the other scenarios, detection failure was highest for heavy risk-based surveillance (7.70%) compared to risk-based (0.10%) and random surveillance (0.00%, Table 4). Similarly, the survey probability of detection at Year 1 was also highest for risk-based surveillance (0.80) followed by heavy risk-based (0.74) and random surveillance (0.58, Table 4, Fig. 10C).

Table 4. Model outputs from empirically derived risk distributions.

Model Run	Surveillance Scenario	Detection Time (Years)		Detection Failure (%)	Survey Probability of Detection at Time (Years)			
		Median Detection Time	Interquartile Range		1	5	10	30
Scenario A Shipping Risk Weighted	Random	0.93	1.47	0.00	0.53	0.98	1.00	1.00
	Risk-Based	0.25	0.47	0.00	0.90	0.99	1.00	1.00
	Heavy Risk-Based	0.37	0.94	4.40	0.70	0.89	0.93	0.96
Scenario B Spread Risk Weighted	Random	0.95	1.36	0.00	0.52	0.98	1.00	1.00
	Risk-Based	0.34	0.66	0.30	0.81	0.97	0.99	1.00
	Heavy Risk-Based	0.52	1.85	8.10	0.56	0.83	0.87	0.92
Scenario C Impact Risk Weighted	Random	0.78	1.33	0.00	0.58	0.99	1.00	1.00
	Risk-Based	0.34	0.68	0.10	0.80	0.96	0.98	1.00
	Heavy Risk-Based	0.27	0.63	7.70	0.74	0.86	0.89	

Discussion

Variation in the risk of NIS introduction and establishment between survey sites (NIS risk distribution) and the level of risk focus which the surveillance strategy adopts have important implications for optimising NIS detection. This study shows that the relative performance of surveillance strategies changes with NIS risk distributions derived both theoretically and with model estimates from the SPT model. Generally, under risk- and heavy risk-based surveillance, time to detection was shorter and survey probability of detection was greater than random surveillance for sites with random or exponential NIS risk distributions. For example, assuming an exponential risk distribution, risk-based surveillance detected NIS twice as fast as random surveillance. Risk-based surveillance also had a substantially higher survey probability of detection after 1 year compared to random and heavy risk-based surveillance. This observation generally held for risk and heavy risk-based surveillance for the marine NIS risk distributions derived from the SPT model, with risk-based and heavy risk-based surveillance having the shortest detection times and highest detection probabilities after 1 year across all three scenarios. This is comparable to the performance between risk-based and random surveillance in other studies (Parnell et al. 2014; Martinetti and Soubeyrand 2019; Mastin et al. 2020). There was, however, a trade-off between short detection time and detection failure in some risk-based simulations, particularly for heavy risk-based surveillance for both theoretical and SPT model-derived risk distributions. Heavy risk-based surveillance over-samples the highest risk sites, rapidly detecting NIS at high-risk sites, but failing to detect NIS at lower risk sites, which, while less likely, can occur. This surveillance method could, therefore, allow NIS to spread undetected at lower risk sites. Heavy risk-based surveillance also had a poor survey probability of detection compared to risk-based surveillance at 1 year and had smaller increases over the long term for both theoretical and SPT model-derived risk distributions. However, trade-offs depend on the risk distribution so that it is conceivable that heavy risk-based surveillance may be advantageous at certain risk distributions. For example, heavy risk-based surveillance performed relatively well for the bimodal risk distribution from the SPT model associated with Scenario C (Impact Risk Weighted). One advantage of the simulator is that the effect of any risk distribution can be tested, which was showcased by the SPT model-derived distributions used here.

An over-emphasis on the highest risk sites can, in some instances, lead to a failure to detect NIS with little benefit in terms of reduced detection time. Concentrating on a small number of sites has also been shown to be detrimental by a spatially-explicit plant pathogen model (Mastin et al. 2020). Inclusion of detection dynamics which varied with NIS abundance had little effect on overall detection time for an exponential risk distribution, suggesting conclusions around the optimum risk focus to reduce the detection time were robust to changes in method detection probability over time. Comparably, probability of detection for heavy risk-based surveillance was similar to risk-based surveillance at year 1, but performed relatively poorly to risk-based and then random surveillance at longer time periods, with the exact relationship changing with the detection dynamic. This suggests the relative performance of different surveillance strategies can vary over time. Overall, for the most likely risk distributions, risk-based surveillance provides the best balance between short detection rates, success in detecting NIS and high probability of detection over the short term.

The model assumes that the risk distribution of sites can be effectively quantified to guide surveillance. Typically, this information is uncertain, particularly for newly-recorded and poorly-understood NIS. However, there is often enough data for effective survey design (Koch et al. 2020). Site introduction risk is often driven by assessment of introduction pathway activity level (e.g. Tidbury et al. 2016, 2021). However, for many NIS, attribution of introduction to a particular pathway with certainty is not possible; rather the link between a species and introduction pathway is based on species biological traits, historical introduction events or introduction events in very different locations, as well as expert opinion. Site establishment risk assessment involves consideration of many factors including environmental suitability (Copp et al. 2016 Davidson et al. 2017). In addition, if 'risk' is based on impact on native species, spread between sites or a combination of factors (e.g. in the SPT model-derived risk distributions used here), then there is a requirement to consider other factors. Impact risk factors may include NIS life history traits and potential NIS impacts on native species via predation, competition, transmission of disease, as well as site-specific factors, such as the presence of vulnerable or protected species (Blackburn et al. 2014). However, translating NIS occurrence into impact is challenging and understanding of NIS impact is a significant evidence gap (Crystal-Ornelas and Lockwood 2020). It should be noted that further work is required to fully integrate this broader concept of risk into the surveillance simulator. When applying the simulator to SPT model-derived site risk distributions, we assumed that the combined risk of introduction, establishment, spread and impact would influence the occurrence of NIS. However, factors driving NIS risk of impact are likely to differ from, or lack spatial correlation with, those affecting introduction and establishment. To better implement this in the model, the option to define different distributions for the risk of introduction and establishment and the combined risk of other factors, on which basis sites are sampled, is required. More generally, the fact that the optimal NIS surveillance strategy varied with risk distribution highlights the importance of improving our understanding of NIS risk and the factors which influence it, whether these be introduction, establishment, impact or potential for spread, at different sites.

Sensitivity and elasticity analyses were performed in parallel to allow both the absolute effect of parameter changes on outputs to be examined and the impact of parameter changes to be compared across parameters. These analyses highlighted the key factors which should be considered when designing a surveillance strategy and the utility of the simulator to explore different approaches. For an exponential risk distribution, the number of seed sites, mean visit rate and method detection probability had the strongest effect on detection time, whereas the effects of all parameters on detection failure were more variable. Differential responses of surveillance strategies occurred between risk distributions. When seed site numbers were greater than one, heavy risk-based surveillance performed poorly for time to detection and detection failure, relative to risk-based and random surveillance. It is possible for NIS to establish at multiple sites early in an invasion (Herborg et al. 2003), such that risk-based surveillance would be effective at ensuring NIS detection over multiple sites. The analyses indicated the minimum desirable visit rate was once per year because time to detection and detection failure increased substantially at lower visit rates. At one or more visits per year, heavy risk-based surveillance generally had the most variable detection times and highest levels of detection failure, suggesting that risk-based surveillance would be more efficient at

visit rates greater than one. The sensitivity and elasticity analyses also allow the impact of changes in sampling effort, potentially linked to funding/resource changes, to be determined. For example, a fall in visit rate from once per year to once every two years, would increase the median detection time from 0.3 to 0.7 years and increase detection failure from 0.3% to 1.1%. Method detection probabilities below 0.5 were associated with long times to detection and high detection failures for random and risk-based surveillance, suggesting that this was the minimum desirable method detection probability. However, at or below a method detection probability of 0.3, heavy risk-based surveillance had the shortest detection times, suggesting heavy risk-based surveillance may be advantageous with low detection probability methods. The need for greater focus on a small number of high-risk sites when using low detection probability methods has been shown in spatially-explicit models (Mastin et al. 2020). The details of sampling methods at individual sites, for example, number of sampling replicates, were not modelled in detail here, but variations in protocols and the method used are likely to change the probability of detection. Detection probability can be calculated statistically (MacKenzie et al. 2002) and high detection probability for marine NIS is achievable using eDNA methods (Fonseca et al. 2023). Overall, these results support the assertion that risk-based surveillance would outperform heavy risk-based surveillance. However, this is conditional on the risk distribution, the default parameters and the outcomes which are most important to the survey objectives. These can be varied to be most suitable to the purpose of the surveillance programme using the simulator.

The simulator is an efficient and valuable tool for planning surveillance programmes. While outside the scope of the current study, several opportunities exist for further development into the future. For example, a spatially-explicit model, incorporating NIS distribution and spread of NIS over time, would allow study of how the spatial distribution of sampled sites influences the utility of risk-based surveillance. Spatially-explicit models of pathogen entry and spread have shown that spatial correlations in risk can make it suboptimal to focus on the highest risk sites and a geographic spread of resources to cover all areas of risk is desirable (Mastin et al. 2020). The rationale for not including a spatially-explicit model of NIS spread was that this model is focused on supporting early warning monitoring, when a NIS is likely only present at a small number of sites (but see Herborg et al. (2003)). This is valid given the likelihood of NIS eradication or successful local management is increased when a NIS is detected quickly after introduction, when the population is localised within a small area (Simberloff 2001; Anderson 2005; Olein et al. 2011). The risk of spread may differ to the risk of introduction, with potentially different risk factors and should be calculated independently (Oidtmann et al. 2011; Thrush et al. 2017). This study focuses on exploration of the impact of overall risk distribution on efficacy of surveillance under different risk-based sampling designs. The simulator allows both the introduction and establishment risk to be defined (and can be extended to include other risk components such as impact and spread). In our illustrative application, we assume that sites have the same risk in terms of introduction and establishment. However, differences may be expected which, depending on the specific scenario, could impact the conclusion as to which sampling strategy would be optimal. Extending functionality of the temporal component to include different months or seasons would allow incorporation of temporal variation in NIS risk and detection probability. NIS risk may change temporally with seasons (Faulkner et al. 2016) or socio-economic changes

impacting risk pathways, for example, changes in shipping activity (Ojaveer et al. 2017). Detection probability may vary over time with a species life cycle and sampling should be targeted to periods where detection is most likely (Harvey et al. 2009), although this is challenging when designing a multi-species detection programme if species differ in their life-history and are associated with different pathways. In this study, it was assumed that only a single species was being sampled. Although this is not unrealistic for a targeted species-specific surveillance programme (Gust and Inglis 2006), NIS surveillance campaigns may also be multi-species, particularly as eDNA methods allow the targeting of multiple species of interest from the same sample via metabarcoding using generic primers or multiplex PCR (Fonseca et al. 2023). Incorporation of multiple introduction and establishment of risk distributions into the simulator will allow output for multiple species to be created, enabling exploration as to whether risk-based approaches, based on average multi-species NIS risks, are appropriate for sampling all species. Finally, the current study is only a theoretical framework and would benefit from further validation. Although we have provided a first step in this direction by parameterising the model with model-derived risk distributions for marine NIS around the UK coastline, further work is required using species occurrence data to determine empirical risk distributions and detailed statistical analysis of model outputs would allow full testing of the robustness of this model.

Conclusion

In conclusion, variation in the risk of NIS introduction and establishment and the level of risk focus of surveillance programmes interact to influence the efficacy of surveillance regimes. Assuming a skewed risk distribution, an over-emphasis on sampling high risk sites will be outperformed by a more balanced focus on high as well as lower risk sites. However, the optimum approach is dependent on the NIS risk distribution. The relative risk of sites and other survey parameters, has to be quantified for the optimal surveillance design to be selected. Overall, this study highlights the utility of model-based simulators to guide decision-making in the design of the surveillance of NIS and other hazards.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

The authors see no ethical implications of this research. All data created by the simulator was synthetic. For the data used by the SPT model, open source data or data available on request was used. No personal data was used in this study.






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Author contributions

Conceptualisation: HT. Methodology: HT, NT, TG, RM, MT, IM. Software: RM, TG, NT. Investigation: TG, RM, MT, IM. Writing - Original draft: TG. Writing - Review and Editing: TG, HT, RM, MT, IM. Visualisation: TG, RM, IM. Supervision: HT. Funding Acquisition: HT.

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Data availability

The simulator code is located in Cefas' reproducible research account on GitHub in the "C8389-NIS-surveillance-simulator" repository (<https://github.com/CefasRepRes/C8389-NIS-surveillance-simulator>). The data and outputs, including example mark-down files from theoretical and SPT model runs used in this publication, are available via Zenodo (<https://zenodo.org/doi/10.5281/zenodo.10355963>). For the SPT model runs, the adjusted code is included along with the data.

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Supplementary material 1

Abundance models, exponential growth model, logistic growth model, elasticity analysis, table and figure

Authors: Thomas I. Gibson, Rebecca S. Millard, Isla MacMillan, Nick Taylor, Mark Thrush, Hannah Tidbury

Data type: docx

Explanation note: Abundance Models; Exponential Growth Model; Logistic Growth Model; Elasticity Analysis; **fig. S1**. Spatial representation of the site risk scores output from the Site Prioritisation Tool; **table S1**. Data sources for each parameter used in the Site Prioritisation Tool.

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Research Article

Lodgepole pine (*Pinus contorta* Douglas ex Loudon) invasion in subarctic Iceland: evidence from a long-term study

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Abstract

The North American lodgepole pine (*Pinus contorta*) has been widely introduced globally and is now considered invasive in several countries. It was first planted in subarctic Iceland in the 1950s. Recently, the forestry sector has strongly promoted it as an attractive means of carbon capture to mitigate global climate change. It is now the most extensively planted tree species in Iceland. We describe the expansion of the lodgepole pine from a mid-20th-century plantation in Steinadalur, southeast Iceland, and decadal changes between 2010 and 2021. The extent of occurrence expanded nearly tenfold, with tree number and population density reflecting exponential growth patterns. The lodgepole pine colonised diverse habitats, including native birch woodlands and heathland, and was associated with significant reductions in vascular plant species richness and diversity. We conclude that lodgepole pine has the characteristics of an invasive species in Steinadalur and that this will also apply to many native ecosystems across most lowland regions of Iceland. Our study highlights the urgent need for management strategies to mitigate the long-term ecological impacts of lodgepole pine invasion in subarctic environments.

Key words: Afforestation impacts, biological invasions, Iceland, invasive species, plant diversity, species richness, Subarctic ecosystems



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Introduction

The limited success in reducing greenhouse gas emissions has driven many nations to explore alternative strategies to mitigate global climate change. Large-scale afforestation has emerged as a widely advocated nature-based solution for carbon capture (Andres et al. 2022; Nasi 2022; Portmann et al. 2022). This approach has been adopted as official policy in numerous countries and has spurred extensive private sector initiatives (Seymour 2020).

Afforestation of previously treeless landscapes represents a profound ecological shift, altering key processes such as soil dynamics, hydrology, species composition, plant functional groups, and vegetation structure (Ehrenfeld 2010; Nuñez et al. 2017). To date, carbon-focused afforestation efforts have largely been dominated by monoculture plantations (Seddon et al. 2021; Bukoski et al. 2022), often relying on exotic species, particularly conifers (Tölgyesi et al. 2022). Many of these species could become invasive (Simberloff et al. 2009). For instance, among widely

planted pines (*Pinus* spp.), those most commonly used in plantations are also the species most likely to spread and become invasive (Wyse et al. 2022). Invasive alien species (IAS) are recognized as significant drivers of global biodiversity loss (Isbell et al. 2022) and have adverse effects on ecosystems and human well-being worldwide (Roy et al. 2024). IAS often reduce regional biodiversity and degrade ecosystem services such as water provisioning and erosion control (Simberloff et al. 2009; Andres et al. 2022; Hua et al. 2022).

Predicting the behaviour of newly introduced exotic species can be challenging. However, certain traits—both of invasive species and the ecosystems they invade—are associated with invasion success. Globally, isolated oceanic islands are particularly vulnerable (Pyšek et al. 2020). Species-poor ecosystems, recently disturbed areas, and open habitats are more prone to invasion than species-rich, undisturbed, or closed natural ecosystems (Lembrechts et al. 2016; Beaury et al. 2020; Lannes et al. 2020). Successful invasive species often originate from biodiverse regions where they evolved under intense competition and predation pressures (Fristoe et al. 2023). While the Arctic and Subarctic were historically considered resistant to IAS (Lassuy and Lewis 2013), increasing human activity, trade, and disturbances, combined with rising temperatures, are now exposing these high-latitude regions to greater invasion risks (Wasowicz et al. 2020).

All these vulnerabilities are evident in subarctic Iceland, an isolated North Atlantic island with a species-poor vascular flora. Iceland's only native forest-forming tree species is mountain birch *Betula pubescens* subsp. *tortuosa* (Ledeb.) Nyman (Thórhallsdóttir 2021). Human settlement, approximately 1,150 years ago, led to extensive deforestation, catastrophic erosion, and significant ecosystem and soil loss (Barrio and Arnalds 2022). Forests and woodlands, which may have originally covered over 20–30% of Iceland's 103,000 km², were reduced to just 1% by 1900 (Aradóttir and Eysteinnsson 2005).

Awareness of Iceland's degraded ecosystems prompted the first restoration efforts in the early 20th century (Olgeirsson 2007). Regular planting of exotic trees began in the 1950s, focusing on conifers: *Picea*, *Pinus*, and *Larix* (Traustason and Snorrason 2008; Eysteinnsson 2017). Afforestation intensified in the 1990s with regional initiatives funded by state grants. Today, most projects prioritize exotic conifers over native species. For instance, lodgepole pine (*Pinus contorta* Douglas ex Loudon), Siberian larch (*Larix sibirica* Ledeb.), and spruces: *Picea sitchensis* (Bong.) Carrière and *Picea × lutzii* Little dominate plantings, with lodgepole pine alone accounting for 25% of trees planted between 2016 and 2020 (4.3 million trees).

Despite this large-scale planting, research on the invasiveness and spread of lodgepole pine in Icelandic ecosystems remains limited. Native to western North America, lodgepole pine is a fast-growing, hardy species that thrives in environments suboptimal for many other timber trees (Vacek et al. 2022). It has been widely introduced in Europe, Turkey, New Zealand, and South America, where it is recognized as invasive in some regions (Ledgard 2001; Richardson and Rejmánek 2004; Langdon et al. 2010; Taylor et al. 2016). However, little is known about its invasiveness in Europe, including Iceland (Jacobson and Hannerz 2020).

This study presents the evaluation of the invasive potential of lodgepole pine in Iceland. Using the work of Guðmundsdóttir (2012) in Steinadalur, southeastern Iceland, as a baseline, we conducted a decadal analysis to:

1. Examine the patterns, speed, and extent of lodgepole pine spread beyond plantation boundaries.
2. Assess its impact on vascular species richness and diversity.
3. Assess population densities and early ecosystem impacts in invaded areas.
4. Evaluate whether lodgepole pine should be classified as an invasive species in Iceland.

Methods

The study species

Lodgepole pine is native to the western part of North America, occurring from SW Alaska and Yukon to Utah, Colorado, and the Mexican state of Baja California (Karl 1993). The species has also been intentionally introduced worldwide. In New Zealand, lodgepole pine was introduced for commercial purposes and for erosion control (Richardson 1998). In northern Europe, lodgepole pine was widely planted because it presented a higher yield per ha and faster growth than the native Scots pine (*Pinus sylvestris* L.) (Karlman 1981). In Iceland, the species has been repeatedly imported for forestry since the first half of the 20th century. Trees originating from the Skagway region are now the most widespread in Icelandic plantations (Sigurgeirsson 1988). The Icelandic plants are likely to be hybrids between *P. contorta* Douglas ex Loudon subsp. *contorta* and *P. contorta* subsp. *latifolia* Engelm. ex S. Watson (Rudolf and Lapp 1987).

Lodgepole pine has a wide ecological amplitude, and is well adapted to survive and reproduce in harsh environments (Wheeler and Guries 1982). Within its native range, it grows from near sea level to an altitude of 3,350 m a.s.l., and from the mild but cool and rainy Pacific coast to the cold and continental interior of the northern Rocky Mountains (Critchfield 1957). In its native range, lodgepole pine grows in a wide variety of topographic settings from flat plains to steep slopes and rocky ridges (Pfister and Daubenmire 1975). It tolerates a wide spectrum of soil conditions including both dry and wet, fertile and poor soils and even bare gravel (Despain 2001; Elfving et al. 2001).

The lodgepole pine's ability to thrive across diverse ecological conditions, regenerate post-fire, and rapidly mature early in its life cycle are essential factors enabling it to play a wide array of successional roles (Elfving et al. 2001). On poor soils, the species can become dominant and represent the final climax stage, forming extensive monotypic stands (Timber Management Research Forest Service 1979).

Lodgepole pine has several life history traits that make it potentially highly invasive. These include small seed mass (<50 mg), short juvenile period (<10 years) and short interval between large seed crops. Small seed mass allows larger numbers of seeds produced, better dispersal, higher initial germinability, and shorter chilling period needed to overcome dormancy, whereas a short juvenile period and short interval between large seed crops translate into early and high recruitment (Richardson and Rejmánek 2004). These advantages facilitated the invasion of lodgepole pine in many countries where it had been introduced by humans (Ledgard 2001; Langdon et al. 2010; Jacobson and Hannerz 2020).

Study site and climate

Steinadalur is a short (2 km) but relatively wide (1.7 km) valley about 3 km inland from the coast in SE Iceland. The valley (ca. 40 m a.s.l.) is open to the east but otherwise surrounded by mountains reaching up to 600 m a.s.l. The bottom of the valley is flat and has been filled with sediment (gravel and stones) by the glacial river Kaldakvísl. The surrounding mountains are largely covered by birch woodland (*B. pubescens* subsp. *tortuosa*) to an elevation of about 200 m a.s.l. The higher parts of the slopes are mostly dominated by heath and grassland vegetation, which is also patchily present in the lower parts of the valley (Fig. 1).

The plantation in Steinadalur consisting of lodgepole pine and Sitka spruce (*Picea sitchensis*) was initiated in 1959 and expanded to ca. 0.02 km² in 1961 (Guðmundsdóttir 2012). The first records of lodgepole pine spread beyond the original plantation date from 1985 (Guðmundsdóttir 2012).

There is no weather station in Steinadalur, but climate stations are located 45 km to the SW (Fagurhólsmýri) and 40 km to the NE (Höfn) and the similarity of their climate suggests that they are a good proxy for Steinadalur. The regional climate is highly oceanic with annual precipitation well over 1,500 mm and exceeding 100 mm in most months (unpublished data from the Icelandic Met Office). The average annual temperature (2000–2020) was 5.3 °C and 5.5 °C at Höfn and Fagurhólsmýri respectively, mean temperature of the warmest month was 13 °C and 14 °C, while that of the coldest month was -1.5 °C and -1.9 °C, respectively (see Suppl. material 1). The frost-free period for both stations was ca. 4 months, from June to September. It is almost certain that due to specific orographic conditions in Steinadalur, microclimatic conditions differ from Fagurhólsmýri and Höfn, but we do not expect these differences to be pronounced. The prevailing winds in the Steinadalur valley are from the northwest (Icelandic Met Office 2022).

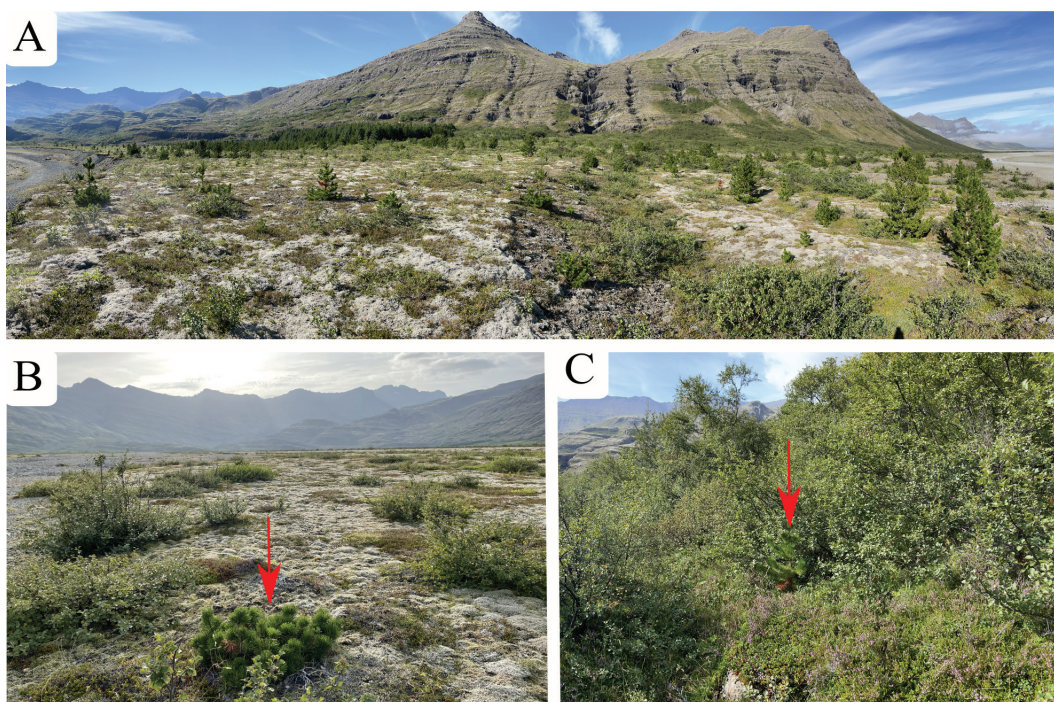


Figure 1. Heath vegetation already colonised by lodgepole pine (plantation can be seen in the distance) (A) and young lodgepole pines (marked with arrows) colonising moss heath in the valley mouth (B) and a birch woodland with dense vegetation cover (C).

Study design and field data

Systematic mapping of decadal-scale distribution changes

Field data were collected in 2010 (Guðmundsdóttir 2012) and 2021, using the same methods to ensure the comparability of data. The methods used were based on Langdon et al. (2010).

Guðmundsdóttir (2012) laid out a series of 24 2 m wide and 100 m long transects radiating at 10 m intervals southwest to southeast from the edge of the 1961 plantation fence. Two transects were excluded as their orientation was miscalculated and they crossed over to neighbouring transects. Each transect was divided into 10 m segments. In each segment, all lodgepole pines were recorded with their GPS coordinates. In 2021, transects used by Guðmundsdóttir were employed to remap lodgepole pine. The transects were lengthened beyond the original 100 meters to cover the newly colonised areas, extending to the banks of the Kaldakvísl River, with an average length of 153 metres. In addition, the total percentage of vegetation cover was recorded for each transect section.

Landscape-scale distribution

In September 2021, the distribution of lodgepole pine within Steinadalur was systematically surveyed and plants mapped using GPS coordinates in order to estimate the extent of occurrence (EOO) of the population (Wilson et al. 2014). The survey boundaries were set by the mountains bordering the valley to the north and south, to the west by the Dalsá river canyon and to the east by the expansive opening of the valley onto the Suðursveit plain. The survey area, approximately 5 km² in size, was systematically covered by two researchers. However, some inaccessible or hazardous areas were excluded from the survey. Due to the substantial increase in pine distribution since 2010 and the aerial extent of the valley, our survey should be regarded as a conservative estimate of the total spread area. GPS co-ordinates were recorded on: Trimble-R8 and GPSmap62s. All collected geographical data were handled in QGIS (QGIS Development Team 2024).

Vegetation sampling

Ten 0.5 × 0.5 m quadrats were randomly placed within three distinct vegetation types: lodgepole pine plantation, uninvaded heathland (located south of the plantation, within the same area as the transects), and uninvaded birch forest. We recorded all vascular plant species present and estimated both total plant cover and cover for each vascular plant species using the Braun-Blanquet scale (Braun-Blanquet 1932). Species concepts and nomenclature follow Wasowicz (2020).

Data analyses

Lodgepole pine density and spread

The density of lodgepole pine was calculated for each 10 m section of all transects and expressed as the number of trees per m². Subsequently, the mean density along all transects was calculated. Changes in density (2010–2021) were mapped and visualised using QGIS (QGIS Development Team 2024).

To calculate the EOO we used the minimum bounding geometry algorithm implemented in QGIS (QGIS Development Team 2024) to calculate a convex hull which covers the whole layer extent for each data set, i.e. collected in 2010 and in 2021 (Wilson et al. 2014). The resulting polygons were used as an approximation of the EOO.

To calculate the rate of spread we converted the outer lines of the convex hull to point layers with the density of 1 point per meter, using the geometry to points algorithm implemented in QGIS (QGIS Development Team 2024). Then the minimum distance from the previous extent geometry for each such point was calculated using the distance to the nearest hub (points) algorithm implemented in QGIS (QGIS Development Team 2024). The resulting distances were used as an approximation of the rate of lodgepole pine spread.

The outer periphery of the polygons, being the result of the previous step of the analysis (see above), were changed into point layers with the density of 1 point per meter, using the geometry to points algorithm implemented in QGIS (QGIS Development Team 2024). These points were used to extract the elevation values from an ArticDEM v3.0 elevation model (Porter et al. 2018). The difference between the highest values of elevation for each dataset served as an approximation of the rate of the vertical spread.

Model fitting

We assessed the relationship between the species' total colonised area and time, and the total number of trees in the transects over time by fitting linear and non-linear models to our observations. Considering the nature of the process (plant invasion) and well-documented spread patterns, the exponential function was likely the most suitable choice. For fitting both linear and exponential models, we employed the nonlinear least squares regression using the *nls* function in R 4.4.1 (R Core Team 2024).

The linear model assumed a constant growth rate over time expressed as:

$$A(t) = a + b \times (t-1985)$$

where:

$A(t)$ is the area occupied by the lodgepole pine (or tree count) at time t

a is the y-intercept, representing the initial area in 1985,

b is the slope of the line, representing the rate of change of the plant area over time

t represents the calendar year.

Whereas the exponential model was:

$$A(t) = A_0 \times e^{r \times (t-1985)}$$

where:

$A(t)$ is the area occupied by the lodgepole pine (or tree count) at time t

A_0 is the initial area of spread in 1985

e represents Euler's number

r is the growth rate

and t represents the calendar year.

The fitting process involved optimising the model parameters to minimise differences between predicted and observed values. Model comparison was performed using three metrics, i.e. residual standard error, variance explained and the Akaike Information Criterion (AIC), providing insights into the goodness of fit and model complexity.

Estimation of species richness

Community level (alpha) species richness and diversity were compared for the three different vegetation types (uninvaded birch woodland, uninvaded heath and lodgepole pine plantation). Statistically significant differences between vegetation types were assessed using the Kruskal-Wallis test and pairwise comparisons using Dunn's test with Bonferroni correction for p-values, at $\alpha = 0.05$.

Results

Decadal changes in EOO and population growth

The extent of occurrence (EOO) of lodgepole pine in Steinadalur expanded nearly tenfold over just a decade, growing from 0.25 km² in 2010 to 2.39 km² in 2021 (Fig. 2). Over the same period, the number of lodgepole pine individuals recorded across 22 transects increased dramatically, rising from 429 in 2010 to 3,315 in 2021—an almost eightfold growth. Similarly, the average density of lodgepole pine across all transects rose by over sevenfold, from 0.06 plants/m² in 2010 to 0.46 plants/m² in 2021. Original data and fitted curves can be found in Suppl. material 1.

The extent of occurrence (EOO) and indices of population growth of lodgepole pine (measured by tree count and mean tree density in transects) were analysed using both linear and exponential models. Exponential models consistently outperformed linear ones, showing lower residual standard error, higher explained variance, and lower Akaike Information Criterion (AIC) values (Table 1). Consequently, the observed trends are best represented by an exponential growth model.

The mean annual spread rate of lodgepole pine increased significantly, from 8.5 ± 2.4 m/year during 1985–2010 to 61.6 ± 40.2 m/year between field studies (2010–2021). Local rates of spread also shifted, with minimum rates rising from 3.4 m/year in 2010 to 8.3 m/year in 2021, and maximum rates increasing from 13.4 m/year to 119.3 m/year over the same period (Fig. 3A). Elevational spread also progressed, with the highest recorded elevation rising from 70 m in 1985 to 116 m in 2010, and 170 m in 2021. Meanwhile, the lowest recorded elevations shifted downward, from 44 m in 1985 to 38 m in 2010, and 25 m in 2021 (Fig. 3B).

Spatial patterns in lodgepole pine density (2010–2021)

Lodgepole pine density exhibited clear spatial gradients in both 2010 and 2021, with the highest densities near the original plantation and decreasing with distance. In 2010, peak densities of 0.5–0.6 plants/m² were observed primarily within 100 m of the plantation edge (Fig. 4). By 2021, these densities had increased by an order of magnitude, with plants recorded farther from the edge, often in areas previously uncolonised.

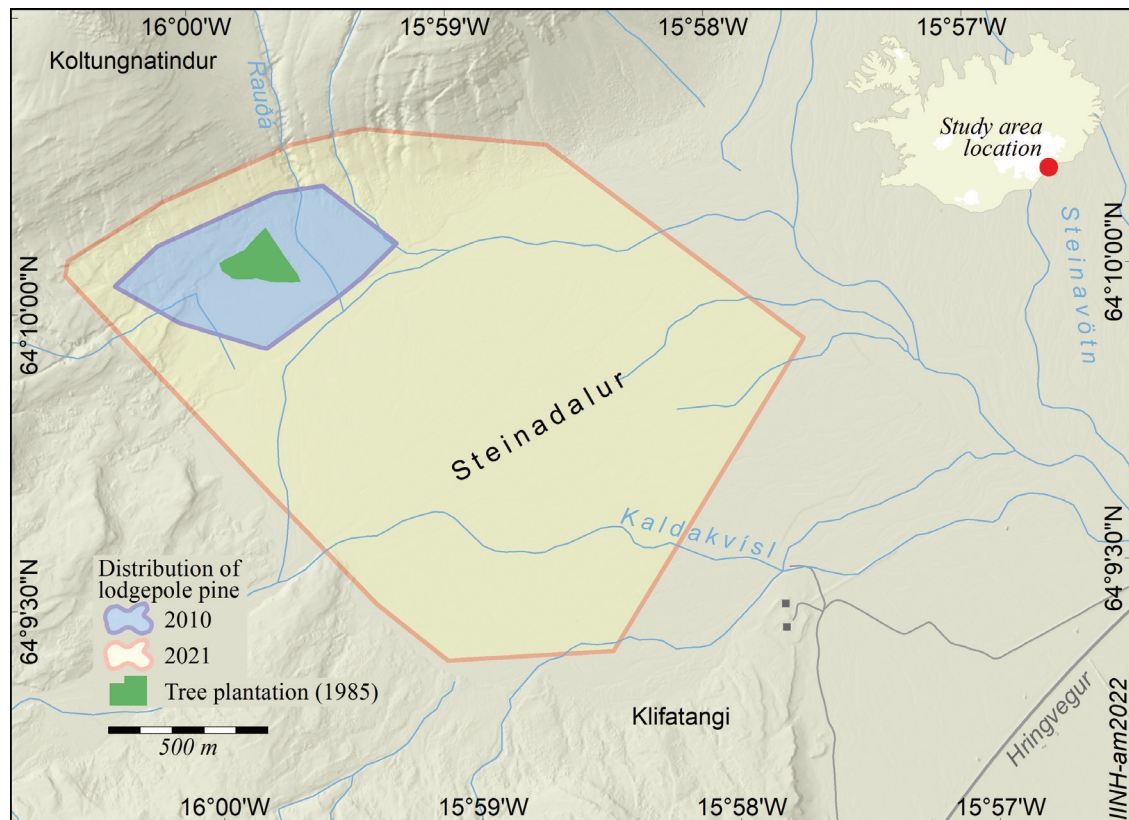


Figure 2. The extent of occurrence (EOO) of lodgepole pine (*P. contorta*) in Steinadalur (1985–2021).

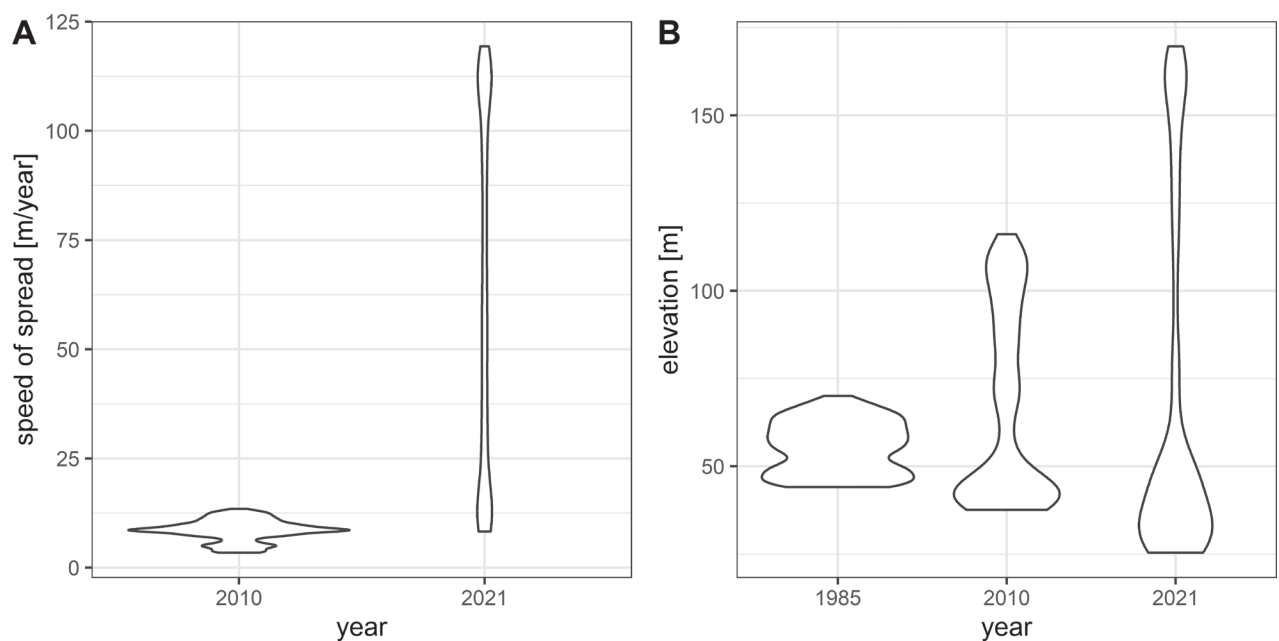


Figure 3. The rate of horizontal (A) and vertical (B) spread of lodgepole pine in Steinadalur (SE Iceland).

Lodgepole pine colonisation occurred across various native habitats in Steinadalur, including dwarf shrub and *Carex bigelowii* heathland, mossy *Racomitrium* grass heath, birch forest, and early-succession open habitats formed by unconsolidated fluvial sediments.

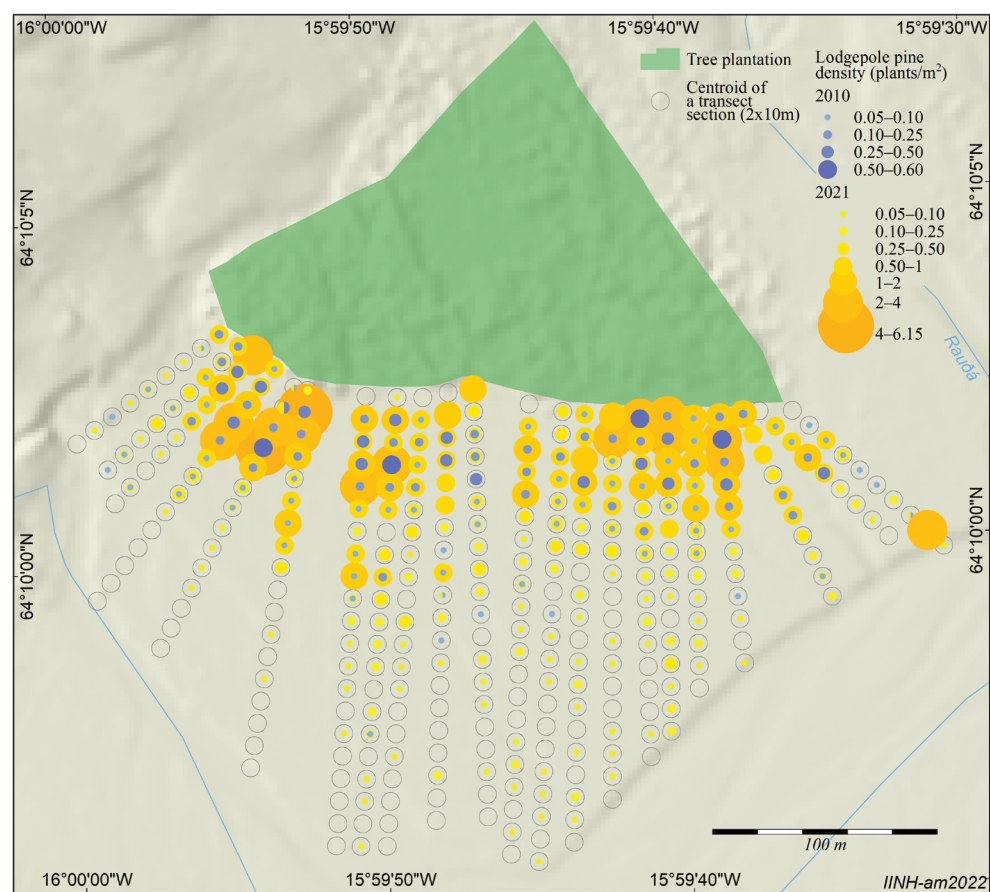


Figure 4. Changes in lodgepole pine densities along the 22 transects in Steinadalur (SE Iceland).

Table 1. Comparison of linear and exponential models using residual standard error, variance explained, and AIC.

		Std. Err.	Var. Expl.	AIC
Area	linear model	1.1	0.64	11.9
	exponential model	1.6×10^{-3}	0.99	-27.5
Number of trees	linear model	1492.0	0.66	55.1
	exponential model	3.1	0.99	18.0
Mean density	linear model	15.9	0.66	1.6
	exponential model	1.8×10^{-5}	0.99	-33.4

Vascular plant species richness and diversity across vegetation types

Vascular plant species richness was lowest in lodgepole pine plantations, with both birch woodlands and heathlands supporting significantly more species (Fig. 5A). Statistical analysis confirmed these differences (Kruskal-Wallis rank sum test: $H = 24.31$, $df = 2$, $p = 5.27 \times 10^{-6}$). Pairwise comparisons using Dunn’s test revealed significant differences between birch and pine ($p = 2.73 \times 10^{-6}$) and between heath and pine ($p = 0.02$), but not between birch and heath ($p = 0.10$).

Similar patterns were observed in Shannon diversity index values, which were lowest in lodgepole pine plantations (Fig. 5B). Differences between vegetation types were statistically significant (Kruskal-Wallis rank sum test: $H = 25.46$, $df = 2$, $p = 2.96 \times 10^{-6}$). Dunn’s test showed significant differences between all vegetation types: birch vs. heath ($p = 0.03$), birch vs. pine ($p = 1.35 \times 10^{-6}$), and heath vs. pine ($p = 0.049$).

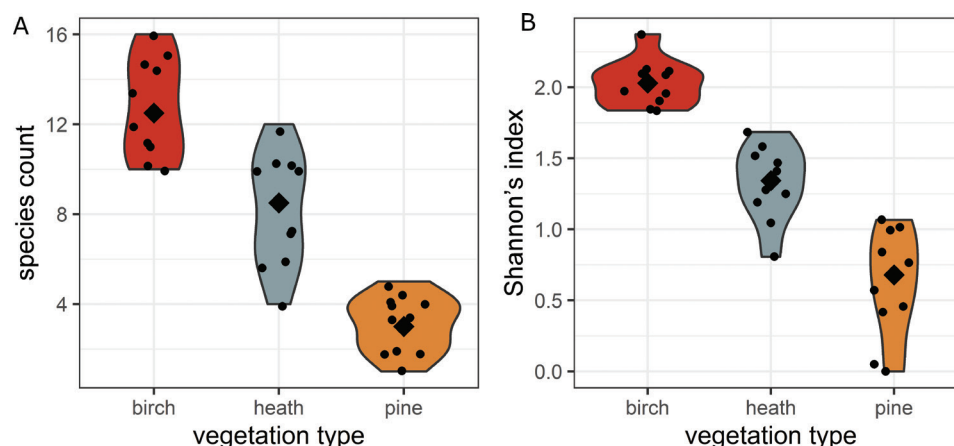


Figure 5. Violin plots showing the number of vascular plant species recorded in vegetation plots (A) and values of Shannon diversity index (B) in three different vegetation types: birch woodland, heath and lodgepole pine plantation. Points denote values of direct measurements, diamonds denote median value for each vegetation type.

Discussion

Patterns of population expansion and growth

The invasion process generally follows a predictable trajectory, irrespective of taxonomic identity of the species (Shigesada and Kawasaki 2001). Following establishment, there is typically a lag period with slow spread, after which the invader enters an exponential growth phase. This expansion continues until available space is saturated, at which point the spread rate levels off (Shigesada and Kawasaki 2001; Arim et al. 2006).

Metrics for lodgepole pine in Steinadalur reflect an accelerating spread, particularly over the last decade. The mean spread rate increased nearly eightfold, from 8.5 m/year over the first 25 years (1985–2010) to 61.6 m (2010–2021). Occupied area expanded almost tenfold, and tree density in belt transects increased nearly eightfold between 2010 and 2021. These rates align with models of exponential, not linear, growth, strongly suggesting that lodgepole pine in Steinadalur has entered the exponential growth phase.

This raises the question: are these patterns primarily driven by Malthusian population growth in unsaturated environment, or do environmental changes, such as climate warming, play a role? Across Europe, the Normalized Difference Vegetation Index (NDVI) has shown a positive trend over the last 30 years (Eisfelder et al. 2023). In Iceland, this trend is pronounced in the west and north but weaker in the east and southeast. These changes are attributed to higher temperatures, increased rainfall, and reduced summer grazing by free-range sheep (Raynolds et al. 2015), although disentangling causal factors remains challenging. Since the 1980s, Iceland's mean temperatures have risen by 0.47 °C per decade, about three times the global average (Hanna et al. 2004; Björnsson et al. 2018).

Steinadalur, located in the southeast of the country, benefits from a milder climate, longer growing season, and higher rainfall than other regions (unpublished data from the Icelandic Met Office). Warm temperatures likely facilitated the lodgepole pine's growth and expansion, and ongoing warming trends are expected to favour it further. Sheep graze in Steinadalur during summer but their impact on the pine has not been documented. The species' altitudinal range, reaching 170 m by 2021, indicates that temperature constraints are unlikely to limit its spread.

Susceptibility of native ecosystems and ecosystem-level impacts

The Steinadalur case study illustrates that lodgepole pine can colonise not only eroded or sparsely vegetated land but also areas with closed vegetation. The lodgepole pine plantation in Steinadalur was established in areas previously occupied by heath or birch woodland. In Iceland, birch woodlands and forests represent the most structurally complex native vegetation. These birch ecosystems vary from old forest fragments with tall, monocormic trees and dense ground layers of graminoids and broad-leaved dicots to open woodlands dominated by polycormic shrub-like birch and dwarf shrubs (Ottósson et al. 2016). According to our results, lodgepole pine is likely to invade most low-stature vegetation types, particularly in warmer lowland regions of Iceland.

Native lodgepole pine forests in North America are characterised by limited undergrowth (Eyre 1980; Perry et al. 2008). When planted outside their native range, lodgepole pine consistently reduces the species richness and diversity of native vegetation (Ledgard and Paul 2008; Urrutia et al. 2013). In New Zealand, vascular plant species richness in grasslands invaded by lodgepole pine declined from 38 to seven species within 20 years (Ledgard and Paul 2008), with none of the remaining species being native. Similarly, in British Columbia, the oldest lodgepole pine forests exhibited the lowest plant species diversity (Sullivan 2004). At the ecosystem level, lodgepole pine invasion constitutes a major state shift. Dense, fast-growing stands drastically reduce sunlight penetration, gradually eliminating shade-intolerant species associated with subarctic heathlands, grasslands, and birch woodlands. In Steinadalur, although based on a small sample size, comparisons of vegetation types strongly suggest significant impacts on native species composition and vascular plant species richness, likely due to light limitation as dense canopies develop, eliminating low-growing and light-demanding species. The significant reductions in species richness observed are clear indications of profound ecosystem changes.

A specific concern in Iceland is the impact of lodgepole pine on native bird populations, particularly wading birds. Iceland is a critical breeding area for waders in Europe (Gunnarsson 2020). Pálsdóttir et al. (2022) documented significant declines in wader densities near plantation edges, likely indicating population losses rather than shifts in habitat use. According to their study, estimated losses from plantations may already amount to tens of thousands of birds, underscoring the far-reaching impacts of tree plantations on Iceland's ecosystems.

In addition to biodiversity loss, lodgepole pine invasion can result in reduced surface streamflow, heightened fire risks, soil erosion following clearcutting, destabilized riverbanks, and a decline in recreational opportunities and grazing land for livestock (De Wit et al. 2001). Managing invasive woody species like lodgepole pine is challenging, with removal often failing to restore ecosystems to their previous state (Panetta 2012; Sapsford et al. 2020). Conifer invasions also alter soil chemistry, hydrology, and fungal communities, potentially increasing soil CO₂ emissions and reducing albedo, which exacerbates warming in temperate and cold regions (Popkin 2019; Nuñez et al. 2021).

Should lodgepole pine be considered invasive in Iceland?

Most criteria for assessment of vulnerability to IAS apply to Iceland: 1) it is an isolated oceanic island (Pyšek et al. 2020; Dueñas et al. 2021), 2) has a natural disturbance regime that periodically creates open ground, and 3) native ecosystems that suffered severe destruction and degradation in the wake of human settlement

(Richardson et al. 1994; Barrio and Arnalds 2022). Finally, the indigenous flora mostly comprises low-growing shrubs and herbs, i.e. growth forms very different from that of the invader (here lodgepole pine, Richardson and Bond 1991). Among the many invasive strengths of lodgepole pine are the high dispersibility of its seeds and frequent copious seed output (Richardson et al. 1994). The future spread of lodgepole pine in Iceland is likely to be significantly accelerated by the widespread practice of establishing numerous small plantation projects across the country's lowland regions.

Lodgepole pine in Iceland fulfils the scientific definitions of an invasive species. Its spread rates, exceeding 100 m in less than 50 years in Steinadalur, and significant negative impacts on biodiversity, comply with IAS criteria (Richardson et al. 2000; Pyšek et al. 2004). Established outside its original plantation for approximately 35–40 years, it has reached distances of nearly 3 km and demonstrated exponential growth. Research is needed to understand its effects on trophic networks, belowground biota, bird and mammal populations, and landscape-scale homogenization. Regional-scale studies are critical, as lodgepole pine appears to be spreading across Iceland, even in colder northern regions (Brynjólfsson 2022).

Implications for management and policy in Iceland

Perceptions of the reality and magnitude of the threat invasive species may pose are known to vary greatly among social groups (García-Llorente et al. 2008), not least when benefitting stakeholders differ from those concerned with negative impacts (Novoa et al. 2024). The failure of many countries to implement successful management and control practices has been attributed to little public and political awareness (Bertolino and Genovesi 2003), and lack of cohesion between scientific researchers, the commercial sector, and policy makers (Stokes et al. 2006). The emerging consensus is that effective management of invasive non-native species largely depends on the active support and collaboration of all relevant stakeholders (e.g. Brundu et al. 2020).

In Iceland, attitudes toward potentially invasive conifers are deeply polarised, with a sharp divide between those anticipating direct benefits and those expressing concerns about environmental impacts. Academics, biologists at state and regional institutes and environmental associations, have warned against indiscriminate use of introduced species and the widespread planting of lodgepole pine (Von Schmalensee 2010; Bjarnason et al. 2023; Jónsdóttir 2023). In contrast, the forestry sector, as well as national and regional afforestation associations have remained staunch advocates of continuing large-scale planting of lodgepole pine and dismiss it as a potential threat to Iceland's biodiversity. The statements and arguments presented in Icelandic media and forestry publications reflect this divide. Claims include rejecting the term “invasive alien species” as invalid and arguing that concerns about invasive species are logically flawed (Sigurgeirsson 2014). Some maintain that lodgepole pine is not invasive (Eysteinnsson 2021), that trees cannot become invasive, or that unwanted trees can be easily removed (Eysteinnsson 2019). Invasion research has been portrayed as pseudoscience driven by nationalism (Sigurgeirsson 2005; Gardarsson 2022; Eysteinnsson 2023). The most pressing task for Iceland is to enhance knowledge through rigorous scientific studies that provide a solid foundation for assessing the risks and long-term consequences of large-scale lodgepole pine cultivation. This evidence should facilitate informed dialogue among stakeholder groups.

Conclusion

This study reveals that lodgepole pine in Steinadalur has entered an unregulated exponential growth phase, replacing natural ecosystems with dense, species-poor woodlands. With no effective competitors in Iceland, lodgepole pine fulfils IAS definitions and poses severe threats to native ecosystems. Addressing its spread requires urgent management and further research on its long-term ecological impacts. As an oceanic, sub-Arctic island with limited native tree flora and degraded ecosystems, Iceland is exceptionally vulnerable to IAS. Indigenous birch forests, covering only 1.5% of Iceland's land area, are unlikely to resist lodgepole pine invasion. The species' ability to form self-perpetuating communities in the absence of native competitors poses a long-term threat to Iceland's ecosystems, landscapes, and biodiversity.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

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Data availability

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

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Supplementary material 1

Climatic diagrams and data for the measured variables, including EOO, tree count, and tree density

Authors: Paweł Wasowicz, Guðrún Óskarsdóttir, Þóra Ellen Þórhallsdóttir

Data type: pdf

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Research Article

Have you seen this fish? Important contribution of stakeholder observations in documenting the distribution and spread of an alien fish species in Iceland

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Abstract

To address the increasing global issue of biological invasions adequate long-term monitoring data is crucial. Due to substantial resource requirements such continuous monitoring remains largely underdeveloped across many countries. In recent years, experiential knowledge of the public, or specific stakeholder groups, has become increasingly popular to gather species' occurrence data. In the context of aquatic alien species recreational fishermen often represent a valuable stakeholder group. Using the case study of alien European flounder (*Platichthys flesus*, Linnaeus, 1758) in Iceland, we explore the benefits of incorporating stakeholder observation-based information with traditionally obtained data on the occurrence and distribution of an alien fish. We compiled records of European flounder reported by the recreational fishing community both when directly approached with an anonymous online survey as well as via social media conversations applying the approach of iEcology. We then contrasted this data with a compilation of European flounder records from databases at the Icelandic Marine and Freshwater Research Institute (MFRI). Our results show that including stakeholder-observation based distribution data in the monitoring of alien species offers significant advantage. While all data sources indicated similar patterns in the spread and distribution of European flounder in Iceland, they differed in the number of unique sites provided as well as their geographic distribution. Combining sources therefore allows to counteract inherent biases present across diverse sources. Our study furthermore indicates that interest in voluntarily reporting European flounder sightings decreased over time, but reemerged when stakeholders and/or the public were presented with an easily accessible opportunity to share information in the form of an online survey. We recommend implementing a monitoring approach for alien species that incorporates diverse sources of information and provides clear venues to report information for the public, and where possible involve stakeholders throughout the entire research process to holistically address biological invasions.

Key words: Biological invasions, European flounder, iEcology, local ecological knowledge, monitoring, recreational fishermen, stakeholder observations



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Introduction

Biological invasions are an increasing global phenomenon (Seebens et al. 2017; Seebens et al. 2020) that can cause severe negative impacts on the recipient environment (IPBES 2023; Schwindt et al. 2023) and result in substantial economic

costs (Cuthbert et al. 2021). Managing the emerging alien species depends on the availability of timely data regarding their occurrence and spread (Groom et al. 2015; Cardoso et al. 2017; Latombe et al. 2017). Unfortunately, systematic management and monitoring of alien species is underdeveloped across many countries (Lehtiniemi et al. 2015; Latombe et al. 2017; Schwindt et al. 2023), often driven by the mismatch between the resources required for adequate monitoring and the funding available to the relevant management agencies and institutions (Piria et al. 2017; Robinson et al. 2020). As the detection rate of new aquatic alien species remains high (Bailey et al. 2020), diverse data sources are increasingly being used to refine distribution estimates (Hargrove et al. 2015; Jarić et al. 2020b; Robinson et al. 2020). These sources include public repositories, unpublished data and interviews with experts, such as those used by Ferreira-Rodríguez et al. (2020) to investigate the historical spread of the Asian clam *Corbicula* sp. in the Lower Danube region. Generally, it is increasingly recognized that collaborations between local stakeholders and researchers can greatly improve the collection of geospatial data (See et al. 2016). Information provided by the public or other stakeholders has often been utilized in invasion science for mapping and monitoring purposes, including studies on plants (Marchante et al. 2016; César de Sá et al. 2019; Gervazoni et al. 2023) and aquatic species (Ferreira-Rodríguez et al. 2020; Herrero et al. 2023).

Under the premise that people either possess valuable information based on their experiences and observations or are willing to learn new skills and contribute to the scientific process, the public can participate at various levels in survey projects (See et al. 2016). Local ecological knowledge (LEK) is defined as knowledge that has established within a specific group of people over time through their interactions with the local ecosystems and/or the utilization of local natural resources. It can be described as a knowledge-practice-belief concept (Olsson and Folke 2001; Löki et al. 2023). At a minimum this entails incorporating stakeholder observations as part of LEK in monitoring activities but ideally, stakeholders are involved throughout the entire research process, allowing them to holistically integrate their knowledge, experiences, and opinions to shape the process and outcome of projects beyond the simple provision of occurrence data. Information on stakeholder observations can be collected via a variety of sources and approaches, such as by directly interacting with target groups through interviews and online questionnaires (Löki et al. 2023) or accessing biodiversity platforms like iNaturalist (Howard et al. 2022). Finally, the emerging field of iEcology, defined as “the study of ecological patterns and processes using online data generated for other purposes and stored digitally” (Jarić et al. 2020a), offers promising, low-cost approaches to collect ecologically relevant data (Jarić et al. 2020a; Jarić et al. 2021). Following the approach of iEcology, data can be harnessed from various sources including social media platforms like Facebook (Pace et al. 2019).

European flounder (*Platichthys flesus* Linnaeus, 1758) is a flatfish species that has been documented in Icelandic waters since 1999 (Jónsson et al. 2001) and is currently classified as potentially invasive (Gunnarsson et al. 2015). The species' native range is in Western Europe ranging from the Mediterranean Sea to the White Sea (Wilson and Veneranta 2019) where it is found in marine, estuarine and freshwater habitats (Skerritt 2010). The European flounder is catadromous (Summers 1979) but plasticity in life history and habitat utilization have been documented in several studies (Daverat et al. 2012; Le Pichon et al. 2014). Previous introductions

of European flounder are known from the Great Lakes in North America, where it was introduced via ballast water but failed to establish (Cudmore-Vokey and Crossman 2000; Ricciardi and MacIsaac 2000). In the years following its first identification in Iceland, European flounder has rapidly spread throughout the country (Kristinsson 2011; Ragnarsdóttir and Metúsalemsson 2020) and while it is mostly encountered in nearshore habitats and estuaries (Henke et al. 2020), it also enters rivers and lakes (O’Farrell 2012; Hlinason 2013). Current information on the distribution of European flounder in Icelandic waters has accumulated through various sources, both formal (i.e. collected by scientific institutions) and informal (i.e. newspaper articles, interviews and similar) (Lúðvíksson 2013; Gunnarsson et al. 2015; NA 2017; Ragnarsdóttir and Metúsalemsson 2020), but there is no ongoing scientific monitoring program for this species. Investigations on the impacts of European flounder are limited but indicate potential competition with and direct predation on native fishes such as European plaice (*Pleuronectes platessa*) (Henke et al. 2020) and salmonids (O’Farrell 2012; Hlinason 2013). Furthermore, recreational anglers in Iceland perceive European flounder to negatively impact their angling activities (Henke et al. 2024). Globally, recreational fishermen have been successfully involved in various studies across different research fields (Löki et al. 2023) and the recreational fishing community in Iceland represents an important stakeholder group that frequently encounters the European flounder (Henke et al. 2024). In Iceland, recreational fishing is popular, with approximately 32.5% of the population participating (Toivonen et al. 2000), and with a revenue of approximately 4.9 billion krona (37 million USD) in angling permits purchased for Atlantic salmon and brown trout (Institute of Economic Studies 2018).

The Marine and Freshwater Research Institute (MFRI) in Iceland conducts many annual surveys targeting native species, for example to evaluate commercial marine ground fish species, and to monitor salmonid stocks in fishing rivers (Jakobsdóttir et al. 2023; Helgason and Bárðarson 2024). Additionally, previous research has shown a high willingness among recreational anglers in Iceland to participate in and contribute to research (Henke et al. 2024). Considering these two aspects, the case study of European flounder in Icelandic waters offers a valuable opportunity to explore the potential of incorporating stakeholder observations in the monitoring of an alien fish species in countries that, despite developed surveying system for many aquatic resources, has no established approach for the monitoring of aquatic alien species.

In the current study we contrast occurrence data of European flounder based on stakeholder observations to data from monitoring and research programs of the MFRI with the goal of evaluating if stakeholder observation-based data can supplement alien fish species monitoring in Iceland. First, we specifically targeted the recreational fishing community in Iceland as a source for occurrence data of European flounder, both with an online survey and with an iEcology approach by mining Facebook posts for location data. Moreover, we used multiple data sources available from the MFRI, marine ground fish surveys and salmonid monitoring, but also logbooks from recreational fishing rivers and voluntary reports of unusual or occurrences of rare fishes received by the MFRI (rare fish database). Specifically, we ask 1) Does European flounder distribution and spread estimates differ by different data sources, that is, regular surveys vs. stakeholder observation-based methods?; and 2) Is stakeholder observation-based data, including MFRI data provided by the public (logbooks and rare fish), a viable option for monitoring alien

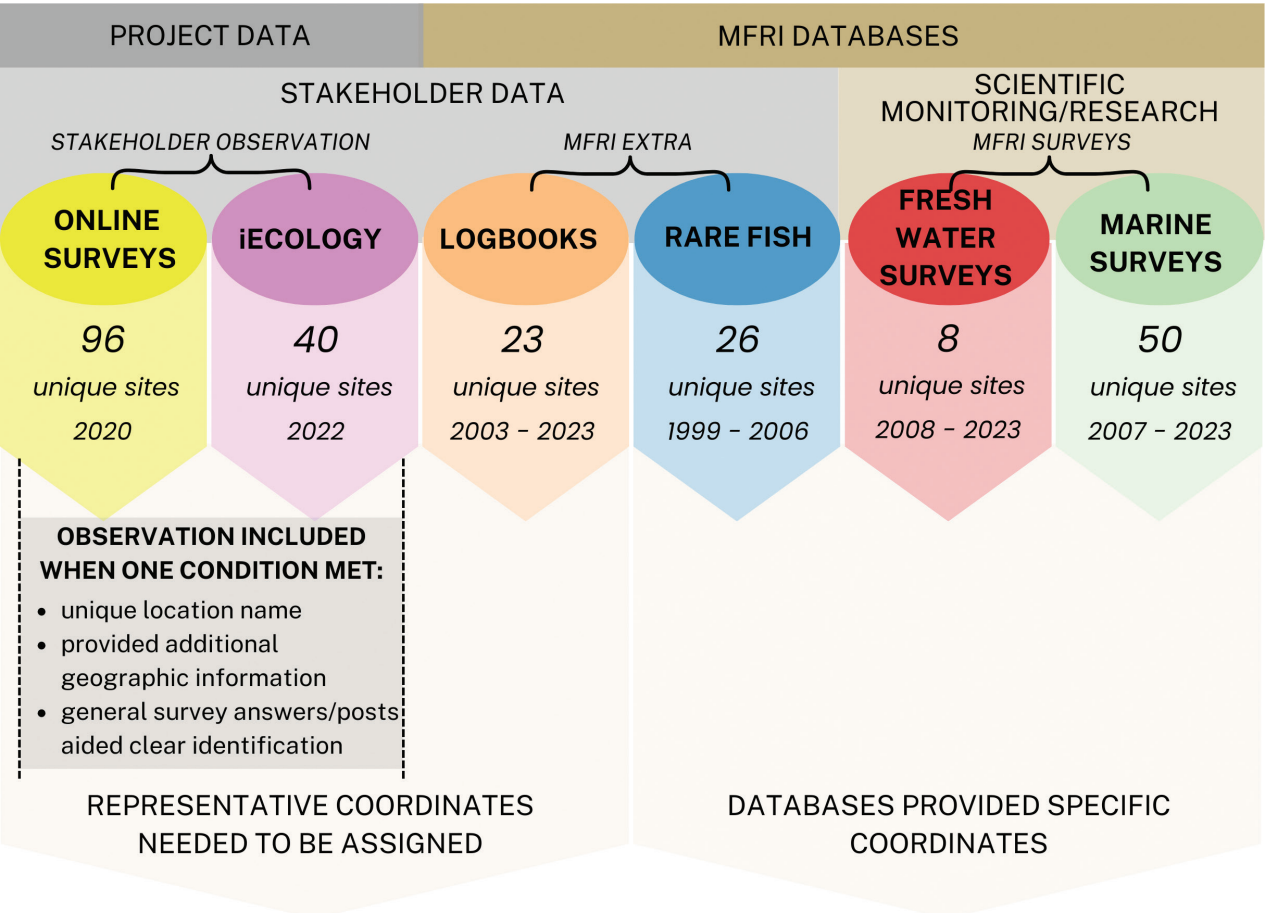
fish species in Iceland? We discuss the findings in the context of strengths and weaknesses of different data sources as a tool for monitoring and how the data availability indicates public and stakeholder willingness to contribute data.

Methods

For the purpose of this study, we have obtained occurrence data of European flounder in Iceland from six different sources (Fig. 1). In the following paragraphs we will introduce each source and provide additional information including how the data for each source was collected, indicate available parameters (i.e. time stamps and exact coordinates), as well as steps that have been taken to address differences in these available parameters. Furthermore, we outline the subsequent steps of analysis.

Occurrence data collected from recreational anglers

First, we conducted an online survey targeting recreational anglers in Iceland to explore their experience with and perceptions of European flounder between October 2019 and June 2020. The anonymous survey, along with information about the aims of the scientific project, was predominantly shared through public Facebook pages of research institutions and a dedicated Icelandic recreational angling Facebook group (15.924 members as of November 2024). We chose Facebook as



EUROPEAN FLOUNDER DISTRIBUTION IN ICELAND
Figure 1. Overview of the six data sources incorporated in the current study.

a tool to reach a wide range of potential participants, as this is a highly popular social media platform in Iceland that around 65% of the population frequently use (Kemp 2024). In November 2019, an article published in a national Icelandic newspaper (Statistics Iceland 2024) that covered the research project (Jónsson 2019) further encouraged participation in the survey. In total, 209 people submitted responses to the survey.

In this survey we asked participants to provide locations where they encountered, either seen and/or caught, European flounder. The occurrence data from the survey was manually reviewed, removing those locations that we could not confidently assign to a specific waterbody. Due to the linguistic characteristics of the place names of Icelandic freshwater systems there are multiple rivers or lakes with the same name that can often only be differentiated when additional geographic indications are provided (e.g. the rivers “Varmá í Mósfellsbæ” and “Varmá í Hveragerði”). For each location provided by survey participants we checked whether at least one of the following conditions applied: 1) it is a recreational angling river/lake with a unique place name not requiring additional geographic information; 2) additional geographic information were provided allowing a clear identification; 3) additional information provided by the participant throughout the survey clearly identify the location such as through information on the region of Iceland they spent most of their time fishing. Locations were removed when none of these conditions were met.

Second, following the concept of iEcology (Jarić et al. 2021), we collected occurrence data from recreational fishermen in Iceland based on conversations within the Facebook group „Veðidellan er frábær...” [The fishing passion is great...], a group highly popular among the target group with 15.4 k members as of 02.05.2024. We evaluated different social media sources for their data availability on European flounder in Iceland as well as biodiversity platforms such as iNaturalist. While most platforms at the end of 2022 indicated a low number of data points relevant to this study (for example only three records had been submitted to iNaturalist prior to 2023) Facebook represented a popular tool among the Icelandic public (Kemp 2024) where European flounder has been frequently addressed. We manually identified Facebook posts and threads mentioning European flounder resulting in 50 recovered conversations occurring between 2013 and 2022 (see detailed information on the extraction and reach of the Facebook group in Henke et al. 2024). From those 50 conversations location reports of either catching or observing European flounder were extracted. Within this manuscript, this data is referred to as iEcology.

As neither the online survey nor the locations extracted from Facebook provided specific coordinates, we determined representative locations for each site. For rivers, representative locations were chosen near the lowest part of the river, for lakes near the mouth of the river through which European flounder most likely entered the lake, and for fjords within major estuaries. These locations were chosen under the assumption that they represent the minimum spread of the species. Where specific location names within habitats were provided, such as fishing beats within rivers, we pooled these locations together to create unique location records.

Occurrence data in the MFRI databases

Data on European flounder was extracted from all available data in the MFRI marine database resulting in European flounder occurrences from major annual surveys, such as, the spring groundfish survey (SMB), the autumn groundfish

survey (SMH), and the gillnetting survey (SMN). The SMB and SMH are annual trawl surveys that sample widely around Icelandic waters in February–March and September–October respectively (Sólmundsson et al. 2022; Jakobsdóttir et al. 2023). The SMN is a spring gillnetting survey sampling inshore waters around Iceland (Bogason et al. 2024). There were also European flounder occurrences from less regular surveys, such as, a discontinued near-shore beam-trawl survey (2017–2022) (Thorlacius et al. 2024) and a demersal seine survey (1995–2013) (Pálsson and Sólmundsson 2017). In addition to these surveys, European flounder was also occasionally reported in marine research projects (Table 1). Survey- and research-based locations extracted from the MFRI marine database were pooled together under the group “marine surveys”.

Similarly, the freshwater MFRI records comprised European flounder location data from two sources. First, we extracted all locations where European floun-

Table 1. The number of unique sites where European flounder has been reported using different data sources. Numbers in brackets indicate the total number of recorded sites. For the MFRI sources the years of the first and the most recent record are listed as well as the number of individual European flounder reported in the records. For the marine surveys data, we furthermore indicated how many of these unique sites were based on research activity or surveys.

Quadrant	SW	NW	NE	SE	Total
Online survey					
Unique sites	36	39	10	11	96
n records	(136)	(121)	(15)	(21)	(293)
iEcology					
Unique sites	21	11	3	5	40
n records	(31)	(30)	(5)	(7)	(73)
Logbooks					
Unique sites	11	10	1	1	23
n records	(17)	(24)	(1)	(1)	(43)
First record	2003	2012	2021	2018	2003
Most recent record	2023	2023	2021	2018	2023
n European flounder	29	434	1	1	465
Rare fish					
Unique sites	15	1	0	10	26
n records	(20)	(1)	(0)	(10)	(31)
First record	1999	2003	-	2000	1999
Most recent record	2006	2003	-	2006	2006
n European flounder	30	1	-	13	44
Freshwater surveys					
Unique sites	5	3	1	0	9
n records	(14)	(3)	(1)	(0)	(18)
First record	2008	2012	2023	-	2008
Most recent record	2023	2023	2023	-	2023
n European flounder	66	5	1	1	53
Marine surveys					
Unique sites	34	13	1	2	50
research	9	4	0	0	13
surveys	25	9	1	2	37
n records	(50)	(25)	(1)	(2)	(78)
First record	2007	2008	2018	2009	2007
Most recent record	2023	2023	2018	2021	2023
n European flounder	370	279	1	2	652

der had been caught in freshwater surveys conducted by the institute. The MFRI sampled widely across recreational fishing rivers in Iceland with the main goal of annual salmonid stock assessment, this sampling is conducted at the same time of the year, usually in late summer. Second, we extracted flounder catches documented in logbooks from recreational fishing rivers, a database maintained by the Freshwater Division of the MFRI. Under the Law on salmon and trout fishing (Act 61/2006), fishing associations in Iceland are required to submit catch information on salmonids in their rivers. While catch information was traditionally submitted in the form of physical logbooks, the option of electronic submissions has been available since 2011 and in 2023 the MFRI established an online form allowing anyone to register their catches.

In addition to European flounder occurrence in surveys and logbooks we used data from the “rare fish database” managed within the Demersal Division of the MFRI. The rare fish database is an ongoing project logging reports and catches reported by stakeholders, often fishermen but anyone can report catches. In 2006, European flounder became part of regular surveying in MFRI marine surveys and was no longer reported to the rare fish database (personal communication Klara Jakobsdóttir, MFRI).

Location data from MFRI surveys provided specific coordinates of catches. For freshwater surveys locations within the same river were pooled together to represent unique sites. As the marine habitat cannot be divided by similar geographic boundaries, locations were treated as unique by default and only pooled when the differences in neither longitude nor latitude between two locations were greater than 0.01 (approx. 1.11 km). Logbook locations did not include specific coordinates and were therefore treated like the locations obtained from the online survey and Facebook. A full list of all data used is presented in Suppl. material 1.

Validating sites reported in the online survey

Three sites from each quadrant of Iceland (SW, NW, NE and SE) that had been reported in the online survey were selected and subsequently sampled using a beach seine (10 m long, 6 mm mesh) to confirm the presence of European flounder. Local landowners and/or river managers were contacted for recommendations on sampling sites to increase the likelihood of accessing likely areas of European flounder occurrence as well as to ensure safety during the sampling process. Where the safety of the scientists could not be guaranteed due to known, strong currents, we selected an alternative site nearby based on local recommendations. The sampling at these 12 sites took place between July and September 2020. Sites where not at least one European flounder was caught were considered as not validated and therefore excluded from further analysis.

Data analysis

All data handling, statistics and figures were done using R (version 4.3.2, R Development Core Team 2023). First, we tested differences in the geographical representation of data sources by comparing both latitude and longitude between all data sources (marine surveys, freshwater surveys, logbooks, online survey and iEcology) using a pairwise Dunn test with Bonferroni correction implemented in the R package `dunn.test` (Dinno 2024).

To compare the annual detection of unique sites between data sources with time stamps available (rare fish, marine surveys, freshwater surveys and logbooks), we fitted a generalized additive model (GAM) to the number of unique sites per source using the *gam()* function of the *mgcv* package in R (Wood 2017). The statistical family was zero inflated Poisson (-0.593, 1.903) and the link function identity. We fitted the model with year as fixed and a smooth term.

$$\text{Individual locations per year} \sim \text{Year} + s(\text{Year}, \text{by} = \text{Source})$$

We furthermore examined how well these four sources documented the temporal spread of European flounder in Iceland. As European flounder was first detected in the southwest and southeast of Iceland (Jónsson et al. 2001), we used latitude to approximate the species' northward spread. We fitted a GAM to the latitude of documented European flounder sites using *gam()* to investigate for differences in the detection of the species' spread between the four sources. The statistical family was Gaussian with an identity link function. The model was fitted with year as fixed effect and a smooth term.

$$\text{Latitude} \sim \text{Year} + s(\text{Year}, \text{by} = \text{Source})$$

For both models the smooth term was Year with source as an interaction term to account for the differences between sources. Smoothing parameters were estimated using restricted maximum likelihood. For the purpose of model diagnostics, we inspected fitted residuals and tested for autocorrelation using the functions “simulate.residuals()” and “testTemporalAutocorrelation()” of the “DHARMa” package (Hartig 2022), respectively.

Results

The online survey (205 participants; see details in Henke et al. 2024) and the iEcology approach returned 97 and 40 individual locations, respectively, with the majority of sites located across western Iceland (Table 1, Suppl. material 1, Fig. 2). In monitoring surveys and research activities, the MFRI recorded 50 unique locations in marine habitats and nine in freshwater habitats, which were all predominantly located in southwest Iceland (Table 1, Fig. 2). European flounder was reported via logbooks for 23 individual sites mostly across western Iceland (Table 1, Fig. 2). There were 26 unique site records stemming from the ‘rare fish’ project and these were widely recorded along the south and southwest of the country but also in the northwest of Iceland (Fig. 2). These records date between 1999 and 2006 and represent the early spread of the European flounder before the species was no longer included in the rare fish database (2006/2007). The percentage of unique sites identified among the total records of European flounder differed widely between sources (Table 1). Among all records stemming from the online survey 32.8% were identified as a unique site. In comparison, the highest percentage was recorded for locations retrieved from the rare fish database, where 83.9% were unique sites.

The various data sources differed significantly in their geographical representation (Fig. 3). The marine surveys reported significantly more locations further west than the rare fish database, the iEcology approach and the online

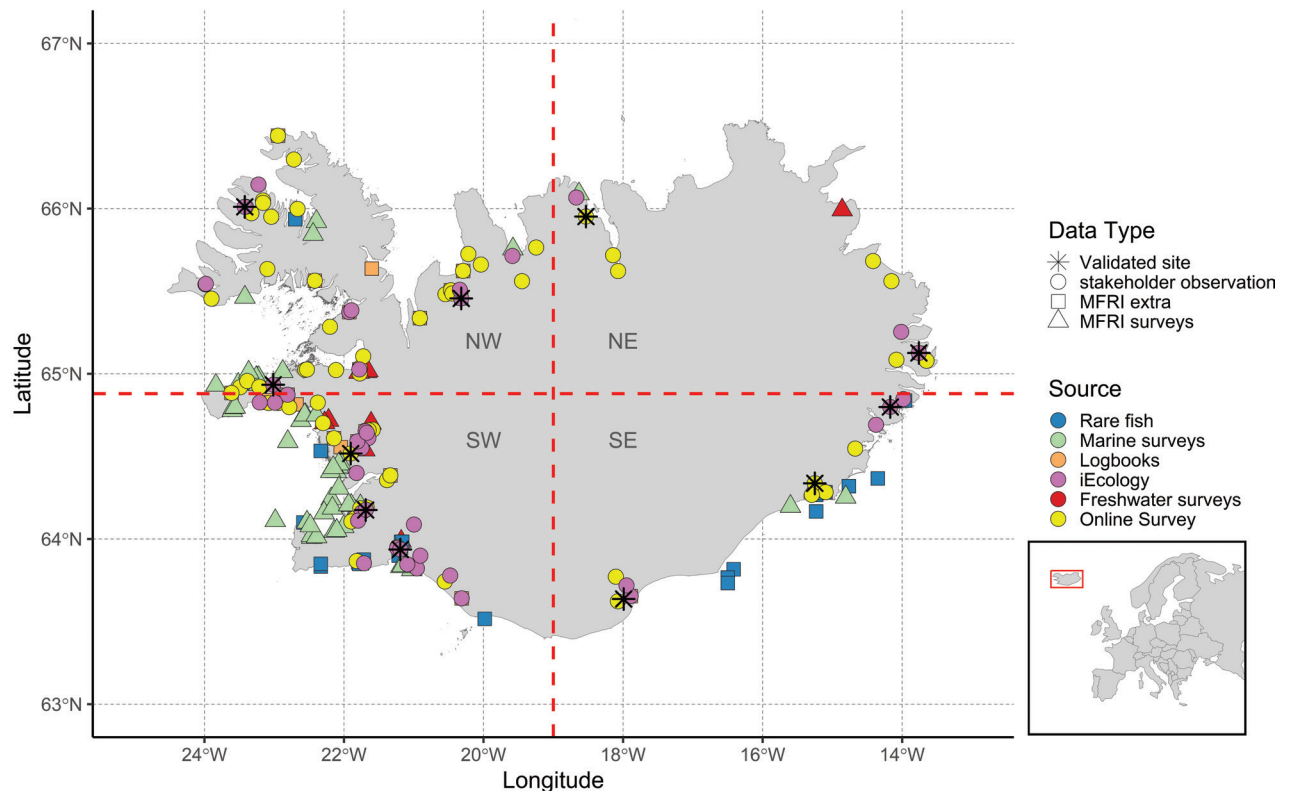


Figure 2. A map of Iceland showing all identified unique sites. For each site the color indicates from the six sources of European flounder locations while the shape highlights the three different data types that we divided the sources into, namely stakeholder observations (Online survey & iEcology), MFRI extra (Logbooks and Rare fish), and MFRI surveys (including both marine and freshwater surveys). Locations validated by sampling are indicated with a black asterisk.

survey (all pairwise Dunn-tests $p < 0.01$). No other pairwise comparisons were significant for longitude. For latitude the marine surveys had a significantly higher representation of southern sites than the online survey (pairwise Dunn test $p = 0.0209$). Moreover, the rare fish database had significantly higher representation of southern sites than any of the other sources apart from marine surveys (pairwise Dunn-test all $p < 0.01$). No other pairwise comparisons were significant for latitude.

Between the four sources that provided distribution data with attributed time information, the annual detection and overall cumulated number of unique sites widely differed (Fig. 4). The GAM smooths showed significant differences between all sources ($p < 0.05$ for all sources, Table 2). New sites recorded via logbooks increased the strongest in 2016 when nine new sites were added (Fig. 4). In the rare fish database, the highest number of new sites ($n = 8$) was recorded in 2002 (Fig. 4). While the number of new sites detected in marine surveys peaked in 2017 with 11 new sites, freshwater surveys recorded the highest number of new sites in 2012 and 2023 with three sites each (Fig. 4).

Within each of the four MFRI sources, the first record was documented in the southwest of Iceland but the year of first record ranged from 1999 (rare fish) to 2008 (freshwater surveys) but the pattern (Fig. 5). The geographic distribution of records in the subsequent years differs between sources. Most notably here is that within four years (2000–2003) the rare fish data indicated European flounder distribution ranging from northwest to southeast Iceland (Fig. 5). However, statistical tests revealed no significant differences between sources in capturing

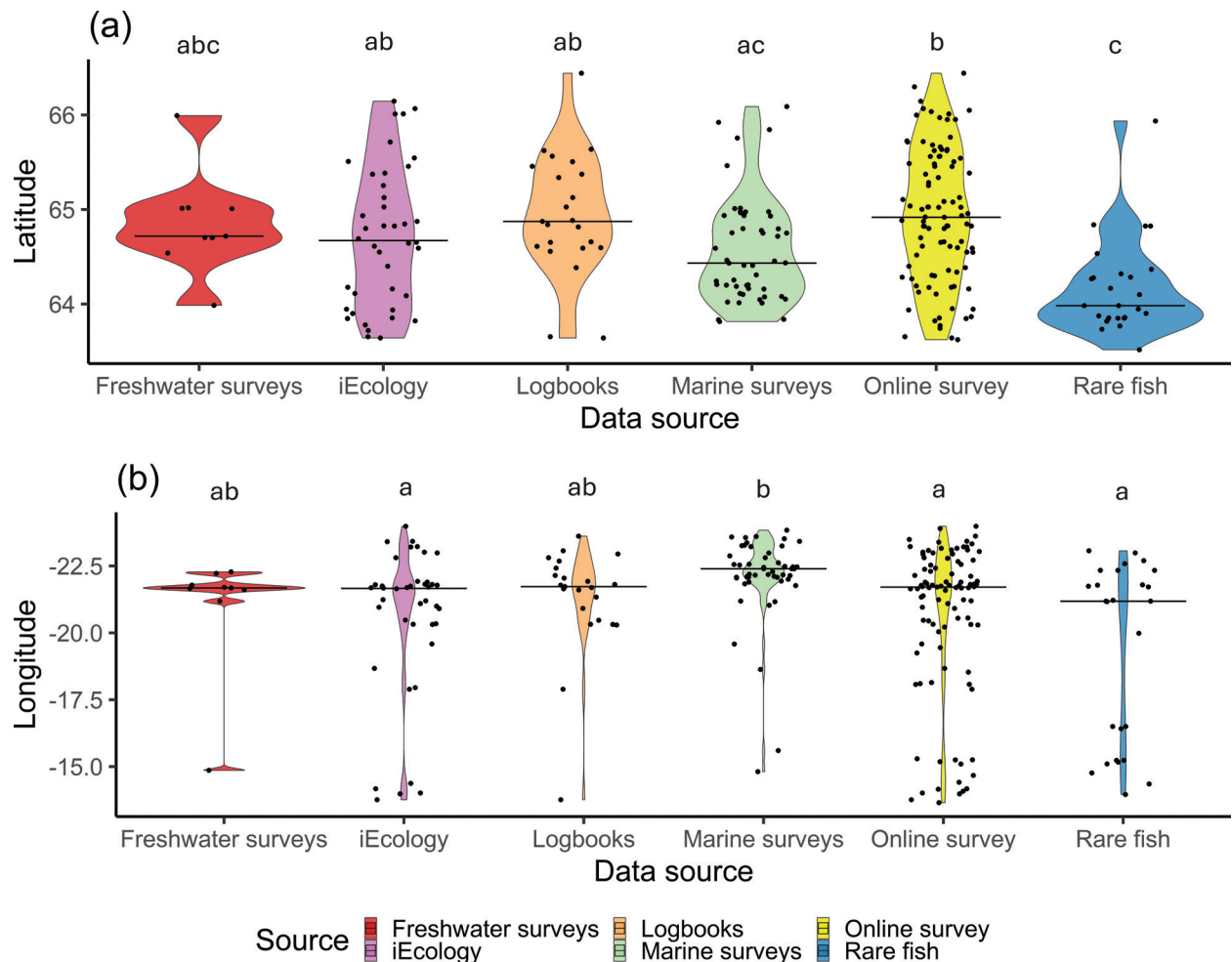


Figure 3. The geographical distribution of reported sites with flounder catches or sightings (note that the figure does not represent the number of fish caught). The figure highlights the geographical biases inherent in each survey method. The black points represent each site. Letters in the graph indicate the results of the pairwise Dunn test with Bonferroni correction where sources that share the same letter are not significantly different.

the northward spread of European flounder approximated by latitude (Suppl. materials 2, 3). It should be noted that the number of available data points for this analysis is low.

The ground truthing of online survey sites confirmed the presence of European flounder in 11 out of 12 sites around Iceland. Despite considerable sampling effort, the sampling at one site in the NE did not capture any European flounder and the record was consequently excluded from further analysis. Interestingly, one additional site in the NE was later confirmed by a MFRI freshwater survey in 2023. Therefore, the lack of validation by independent sampling of the NE sites does not necessarily confirm that European flounder is absent from these sites but is likely rarer in the NE than in other quadrants (Fig. 2).

Discussion

The current case study of European flounder in Iceland shows the benefits of stakeholder observation-based approaches, in addition to existing aquatic surveys, to document both the distribution and the spread of alien fish species.

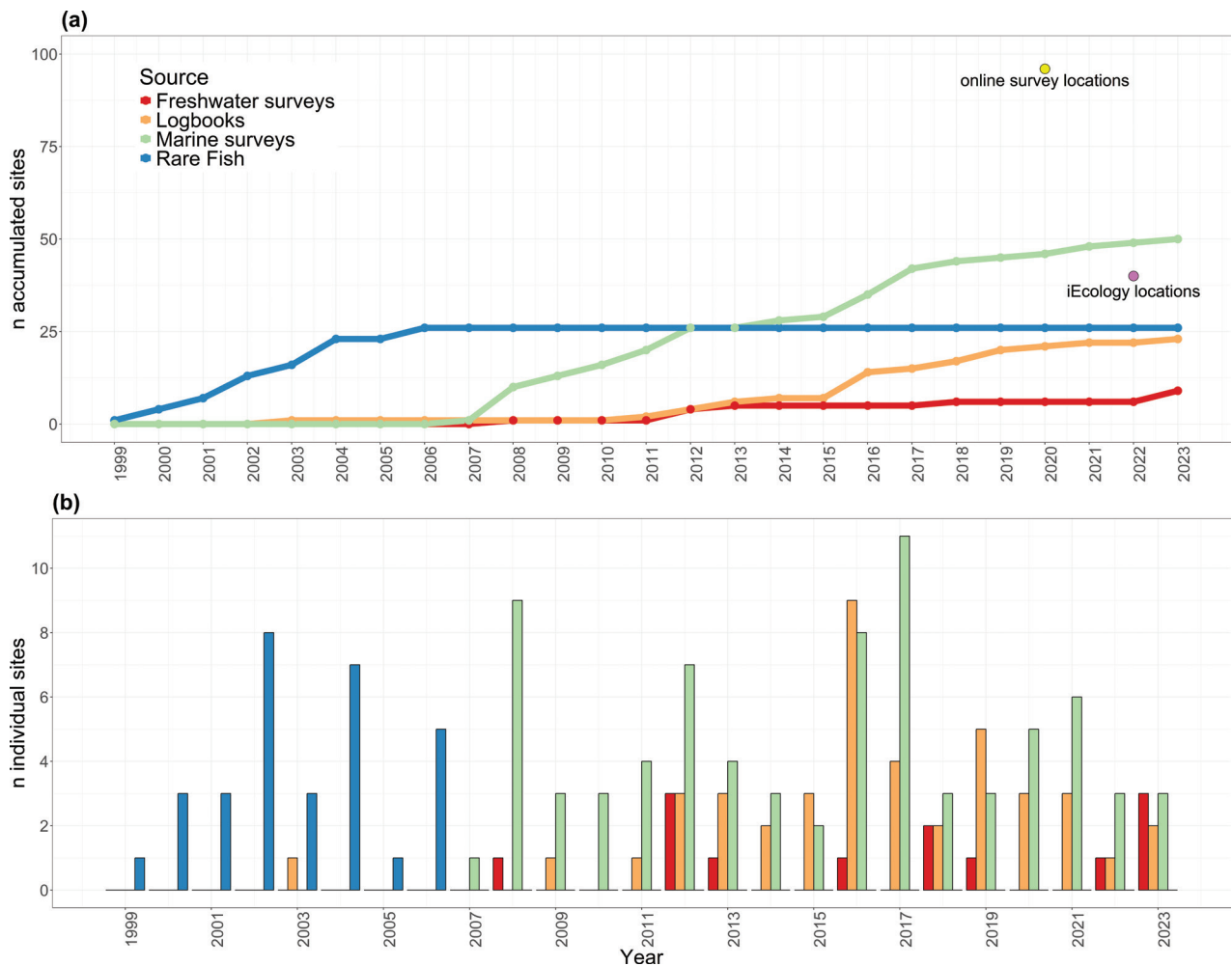


Figure 4. (a) Cumulated number of unique sites per source with time stamped data available. For comparison, the number of sites collected by online survey and iEcology are indicated (b) Barplots showing the number of unique sites recorded annually per source.

Table 2. Estimated parameters based on the generalized additive model (GAM) highlight the differences between data sources in recording unique sites of European flounder in Iceland.

Parametric coefficient	Estimate	Standard error	z value	Pr (> z)	
Intercept	0.000	0.00	NaN	NaN	
Year	-1.163 e ⁻⁰⁴	7.749 e ⁻⁰⁵	-1.501	0.133	
Smooth term	Source	Edf	Ref. df	X ₂	p value
s(Year)	Freshwater surveys	1.000	1.001	6.593	0.0103
s(Year)	Logbooks	3.064	3.757	45.073	< 2 e ⁻¹⁶
s(Year)	Marine surveys	4.888	5.826	86.387	< 2 e ⁻¹⁶

Although the different data sources showed similar distributions and indicated a similar geographic pattern in the spread throughout Iceland, combining sources counteracted the inherent biases of using methods not specifically targeting European flounder. Furthermore, we show that stakeholders are willing to share their knowledge when directly approached with an opportunity such as the online survey, but also based on personal initiative and effort as seen by the occurrences in the rare fish database collected in the early stages of the European flounder spread in Iceland.

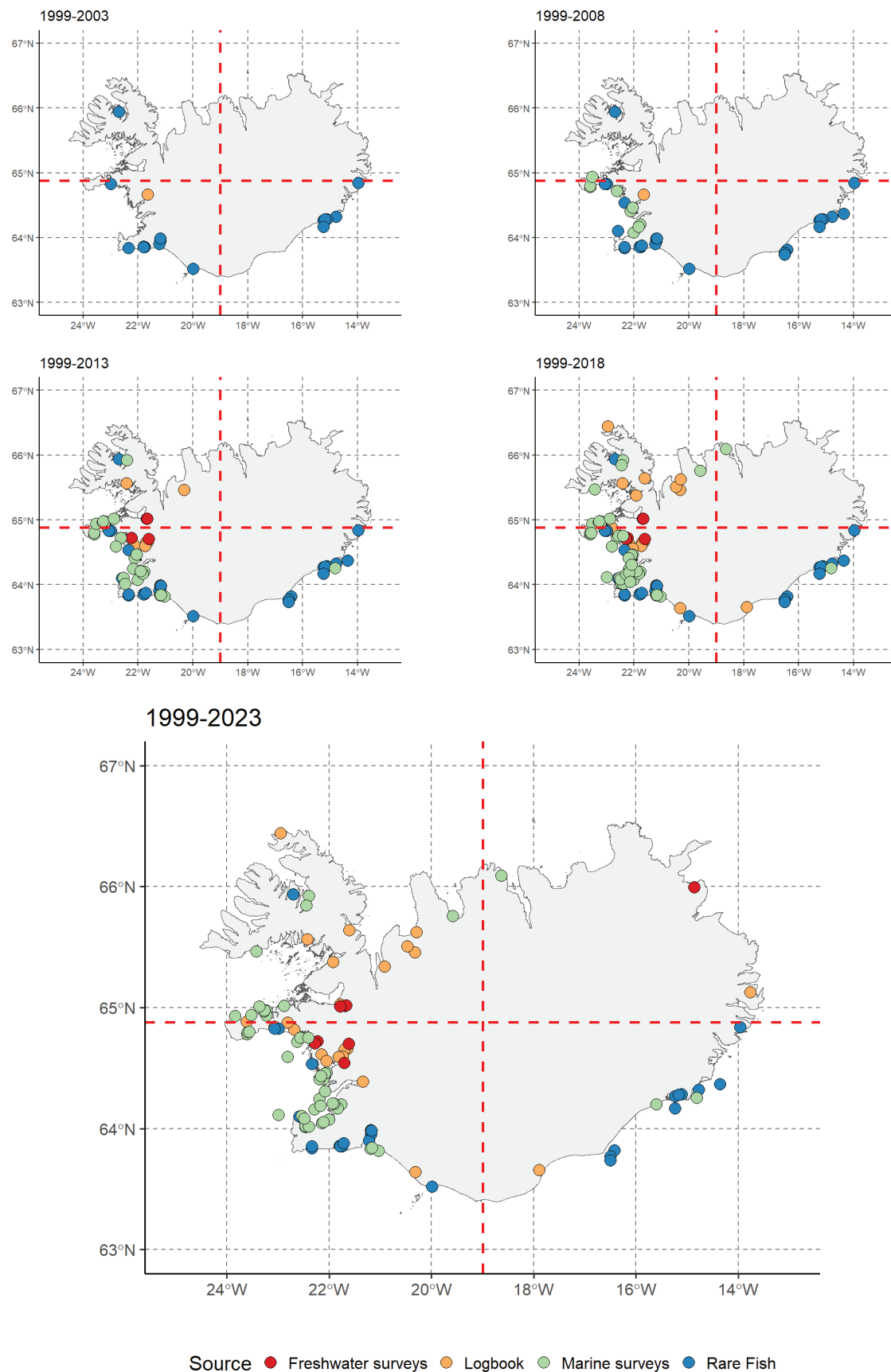


Figure 5. European flounder records mapped for each source that had time stamped data available. The five maps indicate the proceeding spread of European flounder throughout Iceland in five-year increments, with the final map showing all records documented in the MFRI sources until 2023.

Stakeholder observations to document the occurrence of European flounder

The results of the online survey targeting recreational fishermen generated 96 unique locations and therefore provided a higher number of occurrence locations than the previously available survey data based on the MFRI databases, which delivered 50 marine, and nine freshwater locations. Efforts to sample European flounder at 12 representative sites around Iceland named in the online survey showed a high validation rate of over 90% as the sampling at 11 out of 12 sites resulted in at least one individual. While the remaining site was consequently omitted from further analysis, the absence of European flounder in the river Hofsá in northeast Iceland is likely explained by the overall scarcity of European flounder in the northeast region of the country as its presence was confirmed in 2023 during monitoring of salmonid stocks in the same area. The knowledge of recreational fishing communities has previously proven valuable in addressing conservation issues in aquatic environments (Giovos et al. 2019; Löki et al. 2023).

Recreational fishers spend substantial time in the aquatic environments gathering experiential knowledge, beyond what is often available to scientists, and can therefore be more likely to encounter rare or new species (Silvano and Begossi 2012; Löki et al. 2023). The quantitative advantage of stakeholder observation-based data we documented in the current study on European flounder is in accordance with the results of these earlier case studies. While scientific surveying can maximize data quality by strategic sampling plans, stakeholder observation-based data are less predictable. In the current study, stakeholders reported unique sites at a lower ratio than scientific surveys, but the overall higher number of locations provided as well as the confirmation of persistent flounder occurrence provided by repeated reporting of some sites, is advantageous to monitoring activities. An additional advantage of stakeholder observation-based approaches is the increased awareness among stakeholders, which is crucial in addressing biological invasions especially at early stages (Dehnen-Schmutz et al. 2018). The advantages of stakeholder observation-based data were also supported by comparison of the different data sources available within the MFRI. Specifically, large parts of the previously available distribution data on European flounder were already based on stakeholder knowledge, in the form of logbook entries and voluntary submissions to the rare fish database. These submissions were predominantly by recreational anglers and commercial fishermen.

When directly targeting stakeholders or local knowledge holders is not feasible, for example, because of cost, need for prior training etc., iEcology can offer additional approaches to utilize already existing stakeholder observations stored in the form of web-based data such as social media conversations (Jarić et al. 2020a). In the current study the locations scraped from Facebook did not provide the same number of occurrences as the online survey, but it still exceeded the previously documented distribution data based on the freshwater records of the MFRI. Many challenges and limitations have been noted for iEcology approaches, such as, potential spatiotemporal biases and validation of data accuracy (Jarić et al. 2020a; Jarić et al. 2021). However, in the current study 80% of the locations extracted from social media were also named in the online survey, and the approaches produced a comparable distribution pattern, validating iEcology as a tool in the current case study. Overall, in combination with other sources of information and under considerations of potential limitations and biases, social media data can be

of great value when addressing conservation issues (Toivonen et al. 2019) as well as recording new species (Cresson et al. 2021).

Despite the apparent advantages of stakeholder observation-based approaches for documenting European flounder occurrence it should be noted that they provided only presence data while data collected as part of scientific surveys most often provides both presence and absence as well as potential information on size, age, diet and environmental variables at the catch site, i.e. salinity, water depth, etc., as well as co-occurring species. These data are all needed to accurately estimate distribution and habitat suitability as well as the ecological impacts of alien species (Robinson et al. 2020). Conversely, involving stakeholder observation-based data in monitoring activities can enable scientists to obtain large amounts of data in a short amount of time, requiring less resources (Cardoso et al. 2017) and it may be particularly suitable to document early stages and spread of invasions.

Stakeholder observations to document the temporal spread of alien species

In addition to documenting occurrences and spread, public and stakeholder observations are often the first records of an alien species (Thomas et al. 2017; Epanchin-Niell et al. 2021; Kousteni et al. 2022; Pocock et al. 2024). The first official documentation of European flounder in Iceland was based on the submission of a specimen to the MFRI by a member of the public after it was caught at the mouth of the river Ölfusá in southwest Iceland (Jónsson et al. 2001). As shown in this study, stakeholder observation-based sources remain better in reporting European flounder at sites where this species is still rare. Where preventing the arrival of alien species, the most desirable scenario (Browne et al. 2009; Pyšek and Richardson 2010; Schwindt et al. 2023), fails, early detection of alien species becomes crucial (Pyšek and Richardson 2010; Schwindt et al. 2023) but often depends on the premise that the public recognizes and reports unusual observations. Dehnen-Schmutz et al. (2018) identified raising awareness as one of the main topics that should be prioritized in policies addressing biological invasions.

Following the first detection of an alien species, the experiential knowledge of stakeholders can contribute to the reconstruction of the species' temporal spread (Latombe et al. 2017). While there was overall no significant difference in the documentation of the European flounders' northward spread, the voluntary records submitted to the 'rare fish' project already indicated a distribution expansion ranging around half of the country in the early 2000s. While the records of European flounder catches in MFRI's marine surveys generally suggest a similarly fast expansion northward, note that the surveys only officially started recording European flounder after 2006. The recent catch of European flounder during routine freshwater surveying in 2023 provided the first confirmation of the species in the northeast corner of Iceland and the first contribution of formal freshwater surveys to the reconstruction of the temporal spread.

Maintaining stakeholder willingness to report observations

Stakeholder observations-based data highly depends on the willingness of the public and other stakeholders to share their knowledge. The comparably high number of documented locations of European flounder shown in this study indicate that

there is quite some willingness among stakeholders, specifically, the recreational fishing community in Iceland to contribute their experiential knowledge for monitoring purposes. Globally, recreational fishermen have been increasingly involved in management and conservation (Granek et al. 2008) and the results of Copeland et al. (2017), suggest that among other incentives there are social factors providing motivation to get involved.

Our data indicates fluctuations in the number of observations submitted to the MFRI by fishermen, which has also been reported by Cresson et al. (2021), who suggest that the reporting of rare species is not only strongly linked to personal motivation and interest of the fisherman but is also likely to decline once the familiarity with this species increases. The reporting of European flounder catches to the MFRI were mostly initiated by stakeholders' personal motivation although in the years following the first documentation of European flounder news items and reports discussing its status as a new species were relatively common (Henke et al. 2024). The willingness to report could potentially be linked to the general awareness of the public and their interest in the topic of alien species, which for the European flounder in Iceland has been fluctuating over the years (Henke et al. 2024), as well as perceptions of European flounder as novel and "rare" and thereby of interest. In the years following the first official documentation of European flounder in Iceland (Jónsson et al. 2001) when it was still a novelty, occurrences were predominantly recorded as part of the 'rare fish' project, but these reports stopped after 2006. The public's attention towards conservation issues such as biological invasions is generally rather transient (Jarić et al. 2023). The current results suggest that public interest in an alien species is unlikely to be maintained at a level necessary to document spread after initial establishment. However, when stakeholders were approached with an opportunity to share their knowledge and opinions, along with information on the objectives and the anticipated value of their participation, they provided many more locations of European flounder occurrences.

It has been recommended that at a minimum, countries should obtain updated occurrence records of alien species every five years and make these publicly available (Latombe et al. 2017). In this study, we have shown that stakeholder observation-based tools, when designed accordingly, can offer a great contributory source of data where resources for alien species monitoring are limited. Latombe et al. (2017) further suggests that national monitoring of alien species could be improved by building upon already existing structures. In line with previous studies (Jarić et al. 2020a; Löki et al. 2023), we show that there are many ways to collect stakeholder observation-based data, including logbooks, online questionnaires and iEcology. However, there are certain considerations on how to foster a continuous two-way knowledge exchange between stakeholders and scientists to be considered when designing such approaches (Courchamp et al. 2017). Here it can be beneficial to create platforms that are readily accessible and easy to use for the targeted stakeholder group. Recreational anglers in Iceland are required by law to submit catch reports of salmonids (Act 61/2006) and the electronic logbook of the MFRI offers an existing structure to submit catches of Atlantic salmon, brown trout and Arctic charr. A fourth option ("other") allows the records of other caught species, most applicable to catches of European flounder or pink salmon (*Oncorhynchus gorbuscha*), an invasive salmonid species that has shown strongly increasing abundances not only in Iceland but many other northern European

countries (Lennox et al. 2023). Simply providing options specific to European flounder and pink salmon could raise awareness of this information being both officially recorded and important for management, thereby encouraging anglers to report catches. In turn, the resulting data could greatly improve the monitoring of the overall spread of alien fish species in Iceland and provide indications of abundance at very low cost. However, these considerations regarding accessible platforms for data collections should not end at the public and stakeholder level. It should be noted that while we are confident that the underlying data for this study represents the bulk of the available data on European flounder catches in Iceland, we are aware that other institutions and/or scientists may have collected additional catch data. A holistic database that keeps updated records across institutions would greatly benefit not only the monitoring of alien species in Iceland but also other conservation efforts.

Building upon stakeholder observation in the monitoring of alien species

We have shown that stakeholder observations can represent a valuable, complementary source in the monitoring of an alien species. However, considering the context dependency of biological invasions (Robinson et al. 2017; Catford et al. 2022) approaches involving stakeholders need to be carefully designed on a case-by-case basis to appropriately address each invasion individually. In the case of European flounder in Iceland, we selected Facebook over other social media or biodiversity platforms that are more commonly used in similar studies (Pace et al. 2019; Jarić et al. 2021) as Facebook was much more frequently used in this specific case. In addition to choosing the most appropriate data source(s), the reliability of observations regarding the correct species identification needs to be evaluated as this is a crucial aspect of gathering distribution data. In our case, we rated the reliability of stakeholder observations as high based on the fact that European flounder is easily distinguishable from other species in rivers and lakes as it represents the only flatfish species entering these habitats. This was further reinforced by successfully conducted site validation. We recommend that the approach implemented in this study, can be highly valuable and applicable in small nations where resources for formal, scientific monitoring of alien species is limited but collaborations between research institutions, management parties and stakeholder groups are easier to establish.

We acknowledge that, while stakeholder observations are part of Local ecological knowledge (LEK), to holistically incorporate the knowledge of stakeholder groups in the monitoring of alien species, their involvement must be established throughout the entire research process. As the phenomenon of biological invasions is inherently of interdisciplinary and complex nature, embedding LEK in the necessary research and management approaches is becoming increasingly recognized (Caceres-Escobar et al. 2019; McElwee et al. 2020). While the current study shows the benefits of including stakeholder observations in monitoring it does not fully integrate the available LEK. We recommend broadening the approaches to the monitoring of European flounder in Iceland as well as of other alien species, to include LEK where the context allows, for a more holistic understanding of the alien species and their impacts.

Conclusion

Our results show that even with active aquatic surveying, designed to monitor commercially and recreationally important species, there can be a significant advantage to including stakeholder observation-based data sources to monitor alien species. In the case of the European flounder in Iceland, diverse sources based on stakeholder observations, ranging from logbook entries to online questionnaires and social media data, notably improved the information available from surveys carried out by the national marine and freshwater institute. Based on these results and the observation that interest in reporting European flounder as a novel species decreased over time, we therefore recommend monitoring approaches that build upon existing structures providing a clear venue for reporting European flounder occurrences and increased efforts to increase awareness about the issue of biological invasions as well as the value of their contribution. We further recommend expanding on the approach of stakeholder observations and integrating the full scope of LEK embedded in the involved stakeholder group.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: TH, GÁÓ. Data curation: MT, TH, HB. Formal analysis: GÁÓ, TH. Funding acquisition: TH, GÁÓ. Methodology: GÁÓ, TH. Project administration: TH. Supervision: GÁÓ. Visualization: TH, GÁÓ. Writing – original draft: TH. Writing – review and editing: HB, MT, GÁÓ.

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Data availability

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

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Supplementary material 1

Overview table of the underlying European flounder distribution data

Authors: Theresa Henke, Hlynur Bárðarson, Magnús Thorlacius, Guðbjörg Ásta Ólafsdóttir

Data type: docx

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Link: <https://doi.org/10.3897/neobiota.97.132365.suppl1>

Supplementary material 2

Estimated parameters based on the generalized additive model (GAM) highlight the differences between data sources in recording the northward spread of European flounder within Iceland

Authors: Theresa Henke, Hlynur Bárðarson, Magnús Thorlacius, Guðbjörg Ásta Ólafsdóttir

Data type: docx

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Supplementary material 3

GAM smooths with confidence intervals for the recorded northward spread of European flounder in each of the four sources

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Data type: docx

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Research Article

Competitive interactions for food resources between invasive Ponto-Caspian gobies and their native competitors in the context of global warming

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Abstract

Climate warming can modify the process of biological invasions by affecting the outcomes of competition between alien species and their native counterparts in invaded environments. Inland freshwaters are particularly vulnerable to the intensification of such phenomena due to the accumulation of invaders, including thermophilic species that may benefit from warming. We intended to check whether an elevated summer temperature (25 vs. 17 °C) affects the abilities of the Ponto-Caspian gobies to compete for food. These fish are considered temperature-tolerant, highly invasive freshwater fish in Europe. In laboratory experiments, we tested single- and two-species pairs of juvenile specimens of two goby species and their native counterparts from the same ecological guild (the racer goby *Babka gymnotrachelus* versus European bullhead *Cottus gobio*, and monkey goby *Neogobius fluviatilis* versus native gudgeon *Gobio gobio*). The fish competed for food (live chironomidae larvae provided at rates below satiation) for 1 hour at night. We analysed behaviours associated with direct interactions (aggression acts) and foraging activity (time to enter the feeder and the time spent in the feeder). We found that although the gobies did not show higher aggression than the natives, they more actively accessed food compared to the latter, irrespective of temperature. Our results suggest that, in the wild, the invasive fish have a competitive advantage over the native ones due to better resource allocation (gaining food without incurring the costs of aggression) and will maintain this advantage as water warming continues.

Key words: Aggressive behaviour, biological invasions, climate change, food competition, freshwater species, nonnative species



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Introduction

Nowadays, biological invasions constitute a leading threat to global biodiversity (Chandra and Gerhardt 2008; Lambertini et al. 2011; Ricciardi et al. 2013; Reid et al. 2019) with adverse impact on native populations (Dudgeon et al. 2006). This is especially true for freshwater ecosystems exposed to strong human impact (Reid et al. 2019; Bernery et al. 2022), which have been colonised by non-native species to a greater extent than human-affected terrestrial habitats (Dudgeon et al. 2006; Ricciardi and MacIsaac 2010).

Invasions of alien species can be aided by changes in environmental conditions, such as climate warming (Walther et al. 2009; Bellard et al. 2013; Chown et al.

2015; Früh et al. 2017). Climate warming is a global phenomenon that causes multiple changes in the functioning and distribution of organisms, including animals (Isaak and Rieman 2013; Estay et al. 2014; Vázquez et al. 2017). Current scenarios predict an average temperature increase by 2–5 °C (depending on the assumed carbon dioxide emission) by the end of this century (Estay et al. 2014; IPCC 2014). It is worth noting that ectothermic animals are especially sensitive to temperature changes, as their physiology (Vinagre et al. 2014; Marras et al. 2015; Stoffels et al. 2017; Barker et al. 2018) and behaviour (Briffa et al. 2013; Magellan et al. 2019) depend on the ambient temperature. Many invasive species evolved under warmer conditions than their native counterparts, therefore their establishment in novel areas is correlated with ongoing global warming (Hellmann et al. 2008; Rahel and Olden 2008; Jones and Cheung 2015; Hesselschwerdt and Wantzen 2018).

The Ponto-Caspian region constitutes the major donor of alien taxa for European waters (Bij de Vaate et al. 2002; Galil et al. 2007), including several species of goby fish (Gobiidae) (Copp et al. 2005; Roche et al. 2013). The Ponto-Caspian invaders migrate to inland waters of Central and Western Europe through the European river network connected by artificial canals (Bij de Vaate et al. 2002; Pauli and Briski 2018; Soto et al. 2023). Since the 1990s, gobies have quickly increased their ranges throughout Europe (Bij de Vaate et al. 2002; Galil et al. 2007), exerting a strong impact on the environment. The gobies evolved in limans and deltas of Ponto-Caspian rivers where the water temperature reaches 29 °C in July, suggesting the higher upper-temperature tolerance limit of local organisms (Rewicz et al. 2014) compared to central, eastern, and northern European areas. Thus, the invasion of the Ponto-Caspian gobies in Central and Western Europe seems to be linked to the progressive increase in the mean annual temperature (Harka and Bíró 2007).

The success of the Ponto-Caspian gobies is often linked to their effective competition (Kakareko et al. 2013; Jermacz et al. 2015; Grabowska et al. 2016, 2019). Due to their competition with local ichthyofauna, they change the abundance and taxonomic composition of the local benthic fish communities (Gurevitch and Padilla 2004; Kornis et al. 2012; Jakovlić et al. 2015), sometimes contributing to the displacement of native species (Kakareko et al. 2013; Grabowska et al. 2016). The latest studies have shown that the outcomes of interspecific competition between Ponto-Caspian gobies and their native counterparts are variable and can depend on species, size, and reproductive status (Kakareko et al. 2013; Jermacz et al. 2015; Błońska et al. 2016; Grabowska et al. 2016). For example, the racer goby *Babka gymnotrachelus* (Kessler, 1857) revealed greater aggressiveness than the native European bullhead *Cottus gobio* Linnaeus, 1758 of comparable size, being a stronger competitor (Kakareko et al. 2013). On the other hand, the monkey goby *Neogobius fluviatilis* (Pallas, 1814) did not exhibit competitive advantage against European bullhead (Błońska et al. 2016).

Ongoing global warming can reconfigure interspecific interactions between invasive species and their native counterparts (Taniguchi et al. 1998; Oyugi et al. 2012; Carmona-Catot et al. 2013; Ramberg-Pihl et al. 2023), potentially increasing existing and generating new negative effects of invasions (Taniguchi et al. 1998; Carmona-Catot et al. 2013; Ramberg-Pihl et al. 2023). The ability of the Ponto-Caspian gobies to maintain their resting metabolism (SMR) within a range of 17–25 °C at a constant and relatively low level compared to their native

counterparts can be an important trait responsible for their invasive potential (Kłosiński et al. 2024). This way, the gobies can allocate saved energy to interspecific food competition and have an advantage over native species from the same ecological guild.

The tolerance to elevated temperature and competitive efficiency are separate issues which likely interact with each other, but their interacting effects are unknown. Therefore, we aimed to study these interactions experimentally. First, we assumed that interspecific competition between alien gobies and their native counterparts belonging to the same ecological guild is an effect of overlapping food niches (Peiman and Robinson 2010). Second, we assumed that the invasive status of the gobies has been already determined by earlier studies (Copp et al. 2005; Roche et al. 2013; Vilizzi et al. 2019, 2021), and we are looking for their traits contributing to their invasive potential, i.e. their capability to expand and thrive in new areas. In our study, we compared behaviours associated with interference (aggression) and consumptive (exploitative) competition (rapid access to the food source and time spent on feeding) at two different summer temperatures (17 and 25 °C). A temperature of 17 °C reflects the mean temperature recorded in the warm half-year in rivers in central Poland (Marszelewski and Pius 2014, 2016). In turn, 25 °C refers to the mean annual temperature in the warmest month in rivers in central Poland (Marszelewski and Pius 2014, 2016), but is expected to occur more and more often, and for longer periods with ongoing climate change. In contrast to the native species, the invasive gobies had a chance to evolve mechanisms that enabled them to tolerate relatively high temperatures. We hypothesized that the invasive gobies, compared to their native counterparts, are superior in a direct competition for food. Their advantage will be manifested by higher aggression towards their native competitors than towards conspecifics, visiting the food source faster, spending more time in the feeding area, and limiting the access of the natives to the feeding ground. Moreover, we hypothesized that this competitive advantage of gobies over the native species will become more pronounced at 25 than 17 °C.

Materials and methods

Animals

We tested two goby species of Ponto-Caspian origin, the racer goby and monkey goby, paired with their coexisting native competitors: the European bullhead and gudgeon *Gobio gobio* (Linnaeus, 1758), respectively. These two pairs of species were chosen as they co-occur in the same habitats of European freshwater environments sharing similar biology and ecology (Kakareko et al. 2016; Piria et al. 2016; Janáč et al. 2018; Płachocki et al. 2020). Thus, interspecific competition between the alien gobies and their native counterparts can be an effect of their overlapping food niches (Peiman and Robinson 2010). The test species have similar food preferences: benthic invertebrates, especially chironomid larvae (Welton et al. 1991; Declerck et al. 2002; Grabowska and Grabowski 2005; Kakareko et al. 2005; Grabowska et al. 2009, 2024). We obtained juvenile fish from lowland rivers in October–November 2022. European bullhead and racer goby were caught in the River Brda (53°08'52.5"N, 17°58'10.5"E), a tributary of the lower River Vistula, by scuba divers using aquarium nets. At this locality, both species are quite

common and reach similar densities (ca. 60 specimens per 100 m² each) on the river bottom. There is some habitat overlap between small (juvenile) individuals of the two species, with an inverse relationship between their densities, suggesting that competition among them is likely (Kakareko et al. 2016). We collected the fish from four sites (each of about 25 m²) from areas of a depth of ca. 1–2 m and moderate (0.3–0.6 m s⁻¹) water velocity over small stones and gravel, i.e. where their co-occurrence is most pronounced (Kakareko et al. 2016). Gudgeon and monkey goby were collected by electrofishing (EFGI 650, BSE Bretschneider Spezial Elektronik, Germany) in the lower River Vistula (52°26'23.9"N, 19°56'32.5"E). Both species are, in general, common in the river, with the monkey goby considered more abundant than gudgeon in the near-shore fish assemblages (Kakareko et al. 2009; Błażejowski et al. 2022). We collected these species from several sites accessible from the shore by wading, with sandy or sandy-muddy bottoms, and low to moderate flow. Directly after capture, fish were transported to the laboratory (ca. 1–3 h transport time) in polythene bags with oxygenated water. In the laboratory, the fish were placed in 350-L stock tanks with 20–30 individuals of each species per tank, at a temperature measured in the wild (10 °C). After a few days, the temperature in the stock tanks was gradually raised to 17 °C. All specimens used for the tests were of 0+ age. They had no external signs of sexual maturity, thus we did not determine their sex. The stock tanks were filled with conditioned tap water and equipped with aquarium filters, aerators and stony and ceramic shelters, but no bottom substrate. The temperature was maintained by air conditioning at 17 °C. We fed the fish daily with unfrozen chironomid larvae *ad libitum* and uneaten prey were removed from the stock tanks. We exchanged ca. 30% of water volume in the stock tanks once a week. The fish were allowed to adapt to laboratory conditions for at least 1 month before the start of temperature acclimation.

Acclimation procedure to test temperatures

Fish were transferred from the stock tanks to 85-L acclimation tanks in groups of 10–12 individuals, at an initial temperature of 17 °C (as in the stock tanks). The acclimation tanks were filled with conditioned (24 h aged, aerated) tap water and furnished in the same way as the experimental tanks (see below, “Experimental setup”). The progressive adjustment of a temperature up to 25 °C was reached within 8 days using aquarium heaters with an accuracy of 0.25 °C (AQUAEL Ultra Heater 150 W; Suwałki, Poland). During acclimation, fish were fed *ad libitum* once a day with unfrozen chironomid larvae and uneaten prey were removed from the acclimation tanks. Food was delivered with a small amount of water to the acclimation tank on the Petri dish (a feeder placed on the bottom) through the PVC hose and the transparent glass tube. The fish to be tested at 17 °C were transferred from the stock tanks to the acclimation tanks for the same amount of time, but not subject to other temperature alterations. After 8 days in the acclimation tanks, when the temperature reached 25 °C, the fish were transferred to the experimental tanks.

Experimental setup

Experiments were carried out in 27-L tanks (30 × 30 × 30 cm) filled with aged (24 h), aerated tap water. To reduce the effects of handling and visual disturbance on the test fish, the experimental tanks were isolated on all sides by Styrofoam

screens. Each tank was furnished with an aerator, two shelters, aquarium heater (between the shelters), and feeder (Suppl. materials 1, 2). Each shelter was made of a PVC tile leaned against the tank wall at an angle of 49 degrees) in the corner of the tank. The two shelters ensured a refuge for both fish outside the feeding periods to mitigate competitive tensions them. The feeder was located opposite the shelters and heater. The feeder consisted of a Petri dish (attached to the experimental tank bottom with silicone glue), a transparent glass tube (attached to the tank wall with silicone glue, suspended 0.5 cm above the Petri dish bottom) and a PVC hose (coming out of the glass tube on the top and extending beyond the tank) (Suppl. material 1). Food (live chironomid larvae) was flushed with a small volume of water into the Petri dish through the hose and glass tube. The construction allowed us to apply food while minimizing the disturbing effect of the experimenter's presence on the fish. We recorded the experiment using an IP video camera (SNB-6004P, Samsung, Changwon, South Korea) suspended 0.8 m above the water level. Because the test species are nocturnal (see below, "Experimental procedure"), we used infrared lamps (MFL-I/ LED5-12 850 nm, eneo, Rödermark, Germany) for recording in darkness.

Experimental procedure

We took the fish for the research randomly, firstly from the field and then from the stock tanks. The total length of the fish was measured from digital photographs taken during tests using ImageJ 1.53k (freeware by W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA: <https://imagej.net/ij/>). Mean (\pm SD) total lengths (TL) were: 4.68 ± 0.58 cm, 4.87 ± 0.67 cm, 4.70 ± 0.69 cm and 5.31 ± 0.52 cm for the racer goby, bullhead, monkey goby and gudgeon, respectively. Within each species pair, the fish were tested in dyads of similar TL (average difference in TL of 0.10–0.26 cm). Mean TLs of fish in pairs were not significantly different between the species (Student's *t* tests for dependent samples; see Suppl. material 3 for details). The fish were tested either at 17 or 25 °C in (1) single species treatments: two conspecifics, invasive or native; and (2) mixed species treatments: one individual of the invasive species and one individual of the native species. Altogether, we used 71 individuals of the racer goby, 65 individuals of the European bullhead, 78 individuals of the monkey goby and 78 individuals of the gudgeon. In total, we conducted 146 replicates (*n* for a specific treatment = 7–14, see Suppl. material 3 for specific numbers of replicates in particular treatments).

The last feeding took place 40 h before the beginning of the experiment. Two fish (depending on the treatment) were selected from the acclimation tanks and placed in the experimental tank 16 h (at 15:00) before the start of the trial to get familiar with the experimental arena (the adaptation period) (Suppl. material 2). The air stone was turned off before the beginning of the experimental test to prevent water surface movement, which could disturb the video analysis. The tests were always conducted on the following day at 07:00. In the stock, acclimation, and experimental tanks, the photoperiod was set at 12:12 h light:dark cycle with lights on at 10:00 and off at 22:00. Experiments were carried out during the nighttime because the test species are nocturnal and thus their activity (including foraging) is highest at night (Prenda et al. 2000; Erös et al. 2005; Grabowska and Grabowski 2005; Kobler et al. 2012;

Kakareko et al. 2013; Grabowska et al. 2016; Nowak et al. 2019, our preliminary observations). The video camera was turned on at 07:00 and immediately the food (live chironomidae larvae) was delivered manually to the feeder (Suppl. material 2). Fish behaviour was recorded for the next 1 h (07:00–08:00) (Suppl. material 2). This timing was established based on preliminary research, and literature data (Bachman 1984; Taniguchi et al. 1998). Food dose was established as 20–25 mg of live chironomid larvae (2.12–2.65% of the fish weight), which was below the satiation level for one individual (estimated based on preliminary observations). This allowed us to maintain competitive tension between the individuals for limited food resources at the start of the test. Specimens were used only once during the experiments, and subsequently transferred to separate post-experimental tanks with the same water temperature as in the test (Suppl. material 2). After the tests, the elevated temperature in post-experimental tanks was gradually decreased to 17 °C.

Processing video data

Analysis of all the video recordings of fish behaviour was carried out manually, always by the same person, to avoid bias due to differences in the interpretation of fish behaviour. We noted one variable related to aggression and two variables related to foraging: (1) the number of aggressive actions directed towards the opponent, when one fish moved quickly towards the other, which ended in a physical contact between the individuals, such as hitting or pushing (so, the opponents had to touch each other at some moment of the interaction to count the event as aggression). This allowed us to establish clear, strict and objective criteria of aggressiveness, which did not raise any doubts about their correct assessment by the observer; (2) the time to enter the feeder for the first time by each individual; (3) the percentage of time spent by the fish directly in the feeder, which was used as a proxy for food consumption, as it was challenging to observe it directly in darkness. We assume this as a good proxy for foraging, especially in the initial period of the exposure, directly after the food application, when the food was present in the feeder for sure. The animal needed to be present inside the feeder at this moment to have access to the food. In the one-species treatments, because of the visual similarity of the individuals, it was not possible to track them without mistaking particular individuals on video frames. Instead, the two individuals of the same species were tracked together and the final response consisted of summed up and averaged responses of these individuals.

Statistical analysis

We conducted the following types of statistical analyses: (i) comparison between the species within each pair in their single-species treatments (to test differences between the species); (ii) comparison between the species within each pair in the mixed-species treatment (to check which species has an advantage over the other when they are confronted in the same area); (iii) comparison of the behaviour of each species between the mixed vs. single species treatments (to test the impact of one species on the other). Dependent variables tested in the analyses were as follows: (i) the number of aggression events determined in six consecutive 10-min

periods during the exposure (analysed using a General Linear Mixed Model; the use of a Generalized Linear Model designed for count data was not possible due to non-integer data points averaged for single species pairs); (ii) the time spent in the feeder (analysed using a General Linear Mixed Model); (iii) the time to enter the feeder (analysed using a Cox proportional hazard regression to account for the individuals that did not enter the feeder at all). Independent variables were as follows: (i) species (in the comparisons between the species); (ii) treatment (in the comparison between the mixed vs. single species treatments for each species); (iii) temperature (17 and 25 °C); (iv) exposure time counted since the food introduction to the feeder (for the models testing the number of aggression events and time spent in the feeder, a continuous covariate: 10, 20, 30, 40, 50, 60 min), (v) individual pair ID as a random factor (to group repeated measurements for each pair of individuals). Species was a within-subject factor when the species tested in mixed-species treatments were compared to each other. The summary of all the models used in the study is shown in Suppl. material 4. In the above-mentioned models, we included all main effects and interactions and then applied backward simplification of the models by removing non-significant higher-order interactions. To meet the assumptions of the General Linear Model, we tested normality with a Shapiro-Wilk test as well as homoscedasticity with a Levene test. We log-transformed the exposure time, time spent in the feeder and number of aggression events to achieve normality. To disentangle significant interactions between exposure time and categorical factors, we used partial models to check: (1) significances of regression slopes for each categorical level; (2) differences between pairs of significant slopes for different categorical levels; (3) differences between the intercepts (means) of parallel or non-significant slopes for different categorical levels. We conducted all statistical analyses using the SPSS 29.0 statistical package (IBM Inc., USA).

Results

The number of aggression events (racer goby vs. European bullhead)

In all treatments, the number of aggression events exhibited by the racer goby and European bullhead decreased with time (a significant effect of exposure time), but was independent of temperature (Table 1, Fig. 1A–D). The number of intraspecific aggression events exhibited by these fish in the single-species treatments (Fig. 1A) depended on a significant main effect of species (Table 1): the racer goby revealed a significantly greater number of aggression events towards conspecifics (0.8 aggressive events on average during the entire 1-h exposure) than the European bullhead (0.1 events on average).

The numbers of interspecific aggression events displayed by the racer goby and European bullhead in the mixed-species treatment (Fig. 1B, 0.7 events on average) did not differ between the species (Table 1).

The racer goby showed similar levels of intra- and interspecific aggression (Fig. 1C), as shown by a non-significant effect of treatment (single vs. mixed-species) (Table 1). Whereas, the number of interspecific aggression events exhibited by the European bullhead was higher than that directed towards conspecifics (Fig. 1D), as shown by a significant effect of treatment (Table 1).

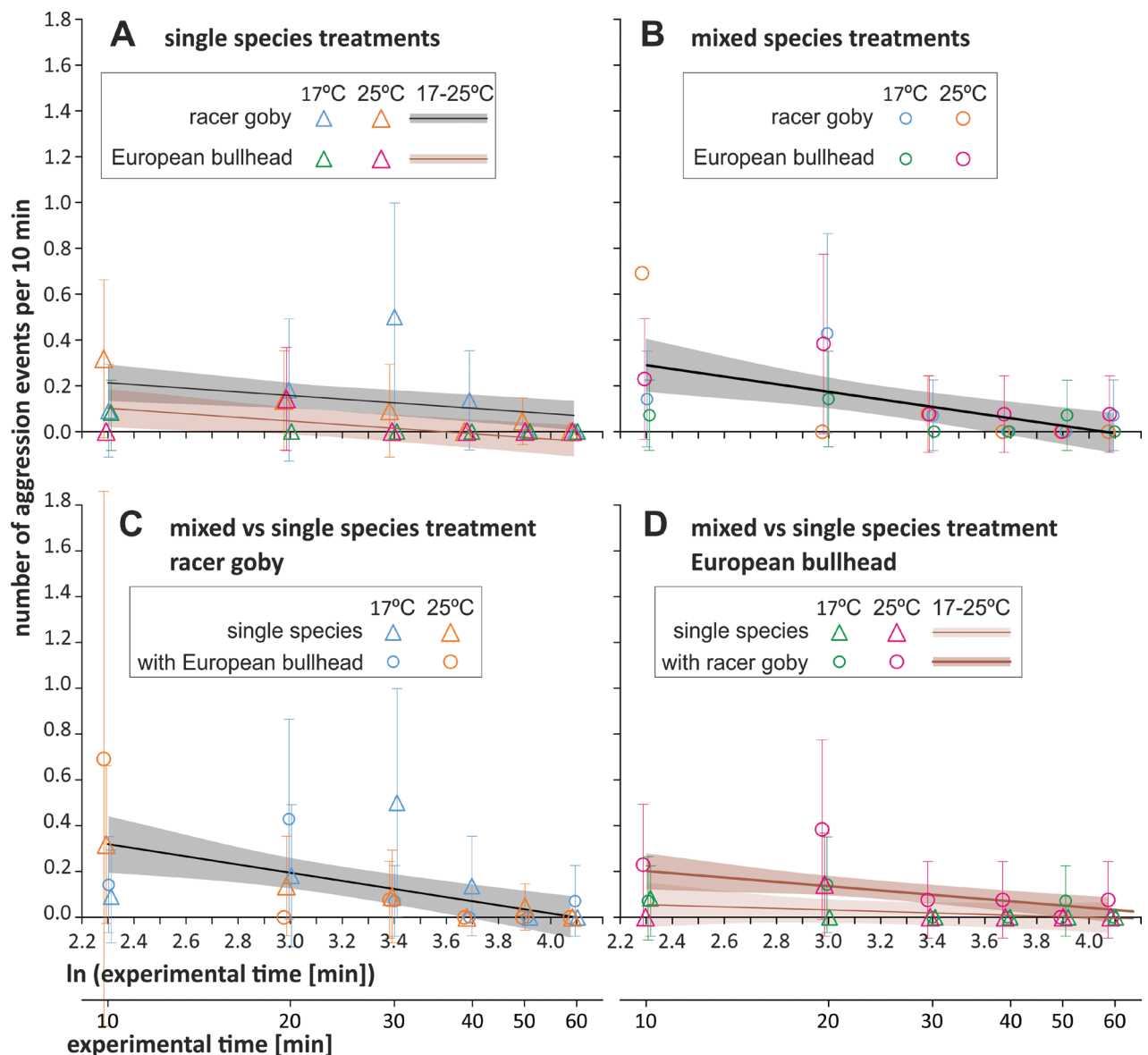


Figure 1. Numbers of aggression acts per 10 min (a single observation period) shown by the racer goby and European bullhead kept in separate single-species treatments (A) or together in the mixed-species treatment (B). Panels C, D present comparisons of the behaviour of the racer goby and European bullhead, respectively, between the single- and mixed-species treatments. Symbols represent raw data (means \pm 95%CI) for each species, temperature and period. Lines are predicted by the models (with 95%CI as shaded areas). Common slopes were predicted for groups of data that did not differ significantly from each other in the models.

The number of aggression events (monkey goby vs. gudgeon)

In the single-species treatments (Fig. 2A), the monkey goby and gudgeon displayed similar levels of intraspecific aggression (0.3 aggression events on average during the 1-h exposure), irrespective of temperature, but decreasing with time (Table 2, Fig. 2A).

The number of interspecific aggression events displayed by these fish in the mixed-species treatment (Fig. 2B) depended on an interaction between species and exposure time, but was independent of temperature (Table 2, Suppl. material 5). This resulted from the significantly greater interspecific aggression of the gudgeon (1.8 aggression events on average during the entire 1-h exposure, including 0.8 events within the first 10 min) compared to that displayed by the monkey goby (0.1 events, all during the first 10 min) at the beginning of the exposure.

Table 1. General Linear Mixed Models to test the impact of treatment, temperature, exposure time and species on the number of aggressive events shown by the racer goby and European bullhead. Non-significant higher order interactions were removed from the models in a simplification procedure.

Analysis	Effect	df	F	P
Racer goby vs European bullhead from single-species treatments	Species	1, 38	8.43	0.006*
	Temperature	1, 38	0.43	0.519
	Exposure time ^C	1, 204	7.85	0.006*
Racer goby vs European bullhead from the mixed-species treatment	Species ^{WS}	1, 295	0.33	0.565
	Temperature	1, 25	0.75	0.395
	Exposure time ^C	1, 295	13.78	<0.001*
Racer goby from mixed- vs single-species treatments	Treatment	1, 46	0.001	0.976
	Temperature	1, 46	0.09	0.766
	Exposure time ^C	1, 244	13.40	<0.001*
European bullhead from mixed- vs single-species treatments	Treatment	1, 43	4.81	0.034*
	Temperature	1, 43	3.81	0.057
	Exposure time ^C	1, 229	8.69	0.004*

^{WS} – within-subject effect, ^C – continuous covariate.**Table 2.** General Linear Mixed Models to test the impact of treatment, temperature, exposure time and species on the number of aggressive events shown by the monkey goby and gudgeon. Non-significant higher order interactions were removed from the models in a simplification procedure.

Analysis	Effect	df	F	P
Monkey goby vs gudgeon from single-species treatments	Species	1, 47	0.11	0.746
	Temperature	1, 47	0.06	0.806
	Exposure time ^C	1, 249	13.73	<0.001*
Monkey goby vs gudgeon from the mixed-species treatment	Species ^{WS} (Spec.)	1, 305	17.58	<0.001*
	Temperature	1, 26	1.06	0.313
	Exposure time ^C (Time)	1, 305	17.28	<0.001*
Monkey goby from mixed- vs single-species treatments	Spec. ^{WS} *Time	1, 305	12.18	0.001*
	Treatment	1, 50	1.88	0.177
	Temperature	1, 50	1.41	0.241
Gudgeon from mixed- vs single-species treatments	Exposure time ^C	1, 264	9.05	0.003*
	Treatment (Treat.)	1, 279	9.42	0.002*
	Temperature (Temp.)	1, 279	1.56	0.212
	Exposure time (Time) ^C	1, 261	19.98	<0.001*
	Treat.*Temp.	1, 279	6.06	0.014*
	Treat.*Time	1, 261	6.36	0.012*
	Temp.*Time	1, 261	1.28	0.259
	Treat.*Temp.*Time	1, 261	5.12	0.024*

^{WS} – within-subject effect, ^C – continuous covariate.

The monkey goby displayed similar levels of intra- and interspecific aggression (single vs. mixed-species treatments) irrespective of temperature (Fig. 2C), but decreasing with exposure time (Table 2).

On the other hand, the number of aggression events shown by the gudgeon (Fig. 2D) depended on an interaction between treatment (single vs. mixed-species), temperature and exposure time (Table 2). The aggression of gudgeon directed towards the monkey goby at 17 °C (2.2 events on average during the entire exposure, including 1.7 events within the first 20 min) was higher than that directed towards conspecifics (0.2 events, all within the first 20 min) at the beginning of the exposure, and decreased later, as shown by its significant slope (Suppl. material 5,

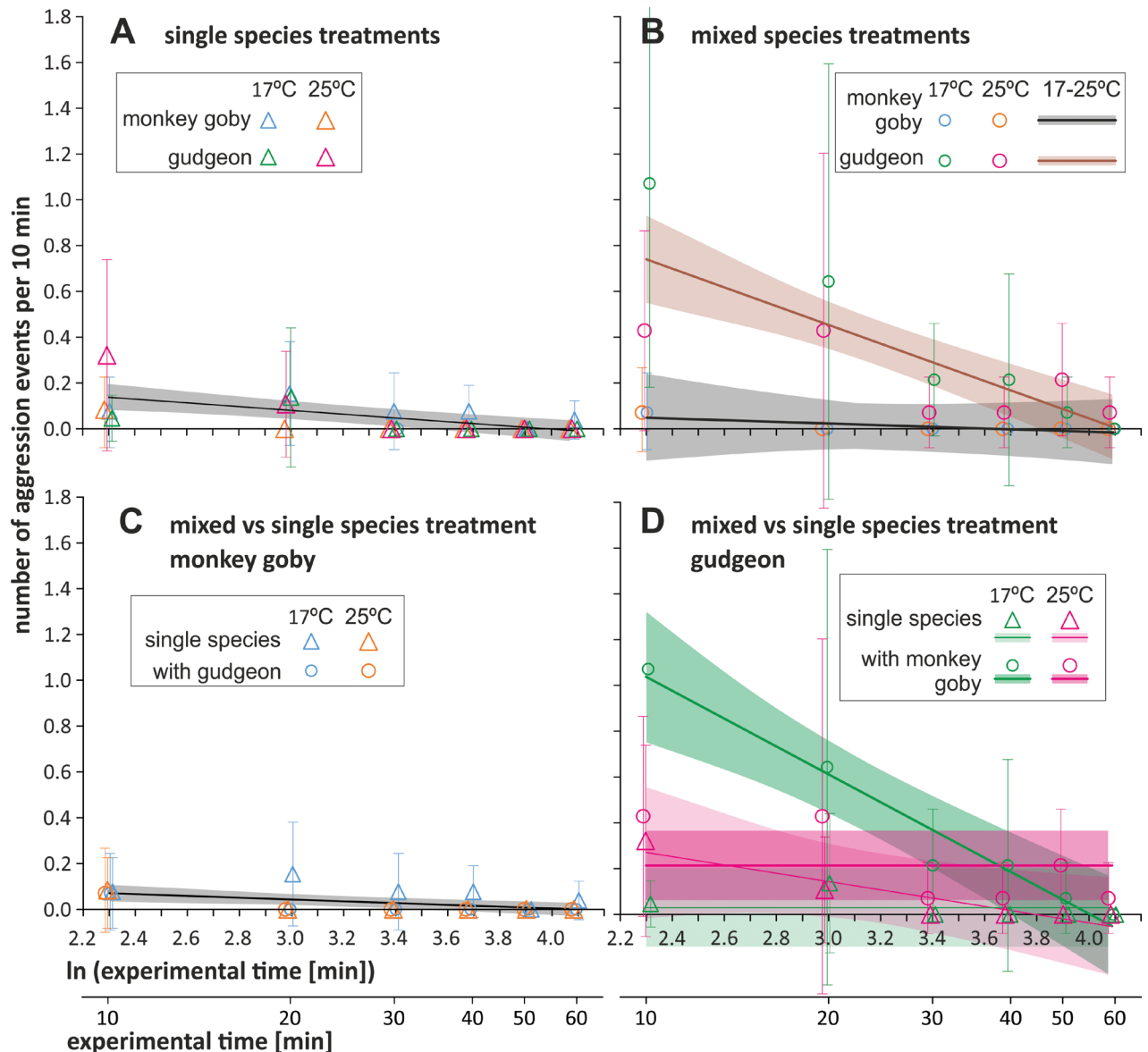


Figure 2. Numbers of aggression acts per 10 min (a single observation period) shown by the monkey goby and gudgeon kept in separate single-species treatments (A) or together in the mixed-species treatment (B). Panels C, D present comparisons of the behaviour of the monkey goby and gudgeon, respectively, between the single- and mixed-species treatments. Symbols represent raw data (means \pm 95%CI) for each species, temperature and period. Lines are predicted by the models (with 95%CI as shaded areas). Common slopes were predicted for groups of data that did not differ significantly from each other in the models. Horizontal lines indicate non-significant slopes.

Fig. 2D). At 25 °C, the gudgeon showed similarly low levels of intra- and interspecific aggression (0.1 events, Fig. 2D).

Time to enter the feeder (racer goby vs. European bullhead)

In the single-species treatments (Fig. 3A) the racer goby entered the feeder earlier (after 2 min on average) than European bullhead (9 min), irrespective of temperature (Table 3).

In the mixed-species treatment (Fig. 3B), the racer goby entered the feeder faster (4.5 min and 13 min at 17 and 25 °C, respectively) than the European bullhead (22.5 min and 36.5 min, respectively), and both species appeared in the feeder faster at 17 vs. 25 °C (Table 3).

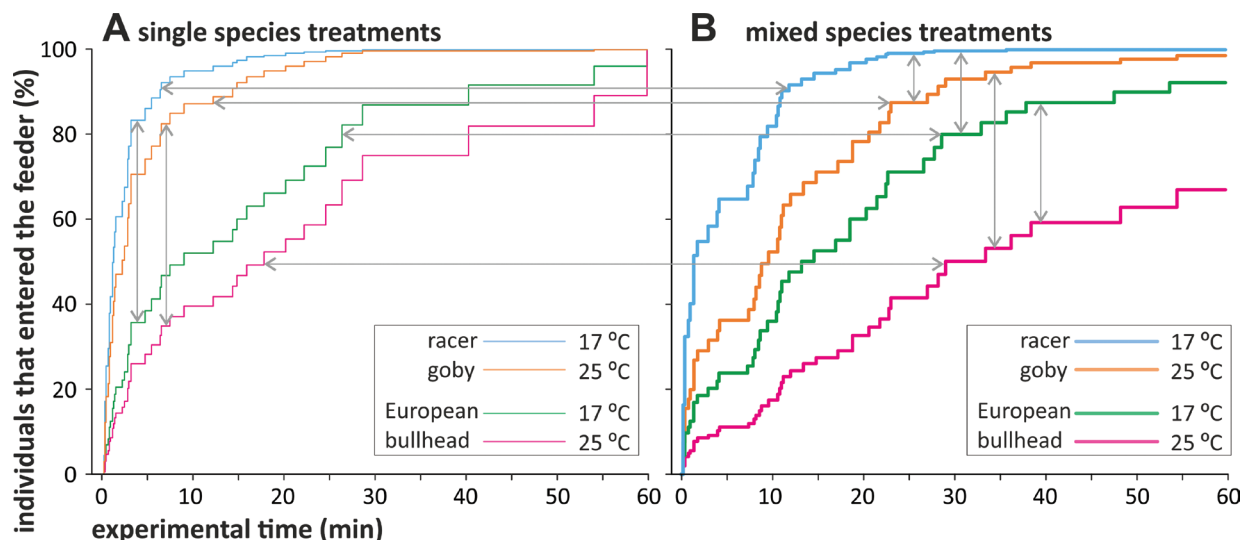


Figure 3. Times to enter the feeder by the racer goby and European bullhead kept in separate single-species treatments (A) or together in the mixed-species treatment (B). Arrows indicate groups significantly differing from each other.

Table 3. Cox proportional hazard regression models to test the effect of treatment, temperature and species on the time to enter the feeder by the racer goby and European bullhead.

Analysis	Effect	df	χ^2	P
Racer goby vs European bullhead from single-species treatments	Species	1	12.92	<0.001*
	Temperature	1	1.16	0.282
Racer goby vs European bullhead from the mixed-species treatment	Species	1	17.54	<0.001*
	Temperature	1	6.99	0.008*
Racer goby from mixed- vs single-species treatments	Treatment	1	4.96	0.026*
	Temperature	1	4.45	0.035*
European bullhead from mixed- vs single-species treatments	Treatment	1	10.58	<0.001*
	Temperature	1	0.63	0.429

The racer goby reached the feeder earlier in the presence of conspecifics than with the European bullhead (Table 3, Fig. 3A, B). The European bullhead also entered the feeder earlier in the presence of conspecifics than with the racer goby, irrespective of temperature (Table 3, Fig. 3A, B).

Time to enter the feeder (monkey goby vs. gudgeon)

In the single-species treatments (Fig. 4A), the monkey goby entered the feeder earlier (9 min and 3.5 min at 17 and 25 °C, respectively) than the gudgeon (21 min and 7.5 min, respectively), and both species appeared in the feeder faster at 25 vs. 17 °C (Table 4).

In the mixed-species treatment (Fig. 4B), the monkey goby entered the feeder earlier (8 min) than the gudgeon (19.5 min) irrespective of temperature (Table 4).

The entry time to the feeder shown by the monkey goby and gudgeon was independent of the species identity of the other individual in the pair (Table 4, Fig. 4A, B).

Time spent in the feeder (racer goby vs. European bullhead)

In the single-species treatments (Fig. 5A), both the racer goby and European bullhead spent more time in the feeder at 17 than 25 °C (3.4 vs. 1.9% of the total exposure time) throughout the exposure time as indicated by a significant main

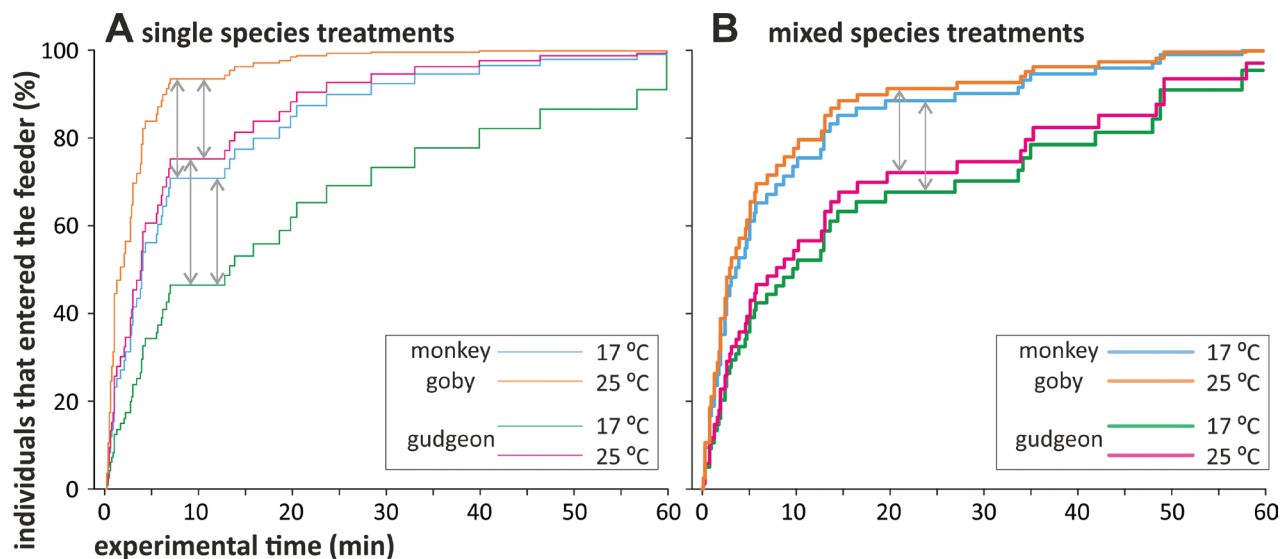


Figure 4. Times to enter the feeder by the monkey goby and gudgeon kept in separate single-species treatments (A) or together in the mixed-species treatment (B). Arrows indicate groups significantly differing from each other.

Table 4. Cox proportional hazard regression models to test the effect of treatment, temperature and species on the time to enter the feeder by monkey goby and gudgeon.

Analysis	Effect	df	χ^2	P
Monkey goby vs gudgeon from single-species treatments	Species	1	5.33	0.021*
	Temperature	1	6.42	0.011*
Monkey goby vs gudgeon from the mixed-species treatment	Species	1	4.74	0.029*
	Temperature	1	0.18	0.671
Monkey goby from mixed- vs single-species treatments	Treatment	1	0.56	0.454
	Temperature	1	0.10	0.754
Gudgeon from mixed- vs single-species treatments	Treatment	1	1.51	0.219
	Temperature	1	7.84	0.005*

effect of temperature (Table 5). Moreover, time spent in the feeder decreased with time (Fig. 5A, Table 5), but differently for each species, which resulted in a significant interaction between species and exposure time (Table 5, Suppl. material 6). The racer goby spent more time in the feeder than the European bullhead at the beginning of exposure (10.0 vs. 2.7% during the first 10 min of the exposure), but not at the end (Fig. 5A).

The presence of heterospecifics in the mixed-species treatment (Fig. 5B, C) did not affect the time spent in the feeder by the racer goby and European bullhead, compared to their behaviour in the single-species treatments, as shown by a non-significant effect of treatment (Table 5).

Time spent in the feeder (monkey goby vs. gudgeon)

In the single-species treatments (Fig. 6A), time spent by the monkey goby and gudgeon in the feeder depended on species*exposure time and temperature*exposure time interactions (Table 6). Time spent in the feeder by both species decreased with time at different rates, depending on species and temperature (Suppl. material 6). At the beginning of the exposure, both species spent more time in the feeder at 25 °C than at 17 °C (4.9 vs. 2.1% of time during the first 20 min of the exposure),

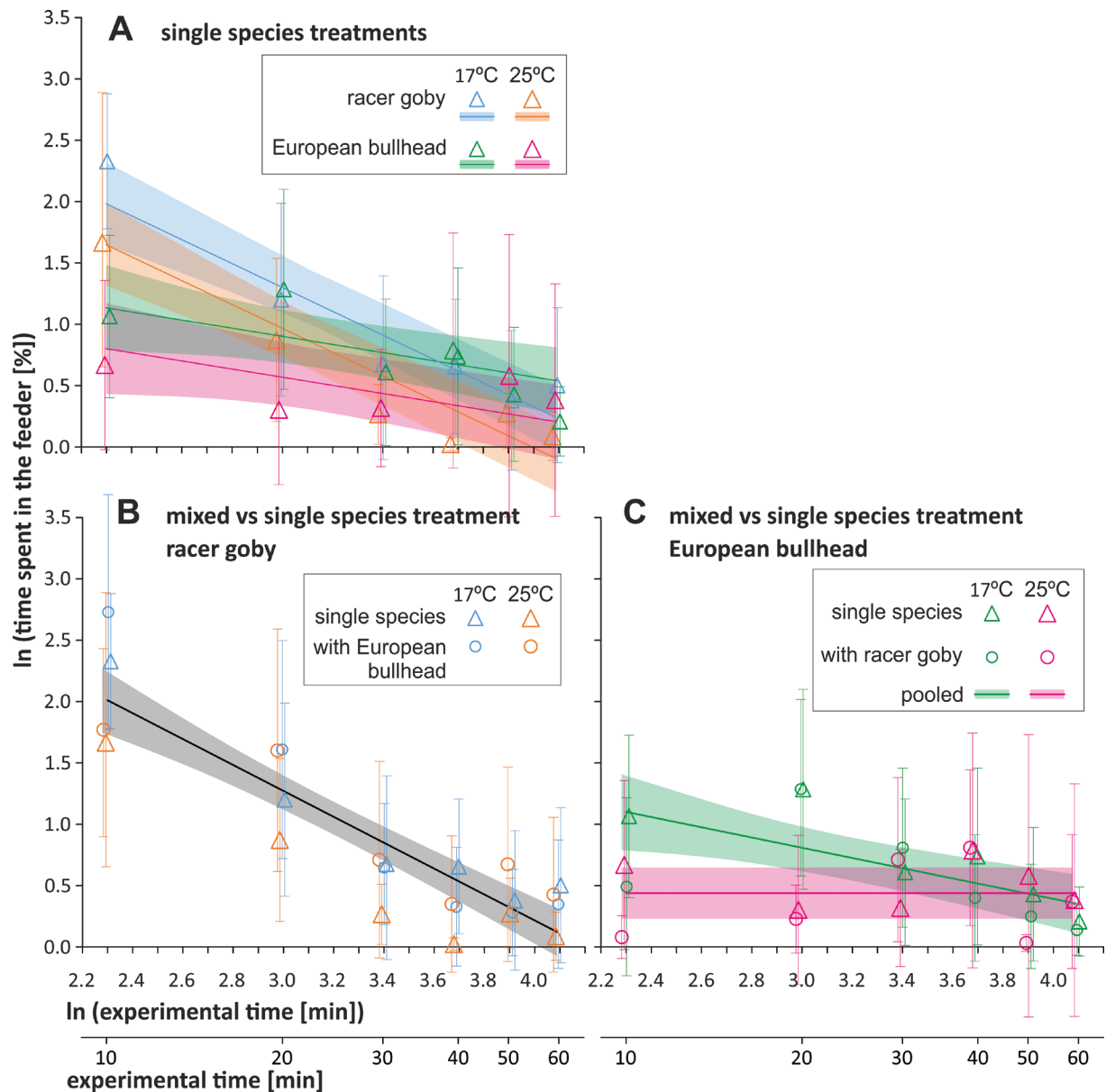


Figure 5. Times spent in the feeder (as percentage of the total exposure time) by the racer goby and European bullhead kept in separate single-species treatments (A). Panels B, C present comparisons of the behaviour of the racer goby and European bullhead, respectively, between the single- and mixed-species treatments. Symbols represent raw data (means \pm 95%CI) for each species, temperature and period. Lines are predicted by the models (with 95%CI as shaded areas). Common slopes were predicted for groups of data that did not differ significantly from each other in the models. Horizontal lines indicate non-significant slopes.

and the monkey goby spent more time in the feeder than the gudgeon (4.7 vs. 2.3% of time during the first 20 min of the exposure).

The feeder was occupied for a longer time by the monkey goby in the presence of the gudgeon in the mixed-species treatment (6.9% of the total exposure time, Fig. 6B) than in the single-species treatment (2%) throughout the exposure duration, as shown by a significant main effect of treatment (Table 6).

Whereas the gudgeon spent more time in the presence of conspecifics than with the monkey goby, but only at the beginning of exposure at 25 °C (6.7 vs. 4.0% of time during the first 20 min of the exposure, Fig. 6C), as shown by a significant interaction between treatment (single vs. mixed-species treatment), temperature and exposure time (Table 6, Suppl. material 6).

Table 5. General Linear Mixed Models to test the impact of treatment, temperature, exposure time and species on the feeder occupancy time shown by the racer goby and European bullhead. Non-significant higher order interactions were removed from the models in a simplification procedure.

Analysis	Effect	df	F	P
Racer goby vs European bullhead from single-species treatments	Species (Spec.)	1, 220	14.44	<0.001*
	Temperature	1, 38	6.74	0.013*
	Exposure time (Time) ^C	1, 203	55.08	<0.001*
	Spec.*Time	1, 203	13.30	<0.001*
Racer goby from mixed- vs single-species treatments	Treatment	1, 46	2.43	0.126
	Temperature	1, 46	2.95	0.093
	Exposure time ^C	1, 244	103.98	<0.001*
European bullhead from mixed- vs single-species treatments	Treatment	1, 43	1.32	0.258
	Temperature (Temp.)	1, 246	9.10	0.003*
	Exposure time (Time) ^C	1, 228	3.19	0.075
	Temp.*Time	1, 228	7.59	0.006*

^C – continuous covariate.**Table 6.** General Linear Mixed Models to test the impact of treatment, temperature, exposure time and species on the feeder occupancy time shown by the monkey goby and gudgeon. Non-significant higher order interactions were removed from the models in a simplification procedure.

Analysis	Effect	df	F	P
Monkey goby vs gudgeon from single-species treatments	Species (Spec.)	1, 264	27.41	<0.001*
	Temperature (Temp.)	1, 264	20.27	<0.001*
	Exposure Time (Time) ^C	1, 247	91.76	<0.001*
	Spec.*Time	1, 247	24.71	<0.001*
	Temp.*Time	1, 247	17.78	<0.001*
Monkey goby from mixed- vs single-species treatments	Treatment	1, 50	8.28	0.006*
	Temperature	1, 50	0.03	0.871
	Exposure time ^C	1, 264	108.14	<0.001*
Gudgeon from mixed- vs single-species treatments	Treatment (Treat.)	1, 290	0.06	0.803
	Temperature (Temp.)	1, 290	8.10	0.005*
	Exposure time (Time) ^C	1, 261	14.47	<0.001*
	Treat.*Temp.	1, 290	3.58	0.060
	Treat.*Time	1, 261	0.02	0.889
	Temp.*Time	1, 261	6.28	0.013*
	Treat.*Temp.*Time	1, 261	4.00	0.046*

^C – continuous covariate.

Discussion

Present work supported the first hypothesis that the non-native gobies are more successful food competitors than their native counterparts. Although the invaders did not consistently outperform the natives in terms of higher aggression, they revealed faster and longer food access compared to the natives. However, the second hypothesis was not confirmed. The effect of an elevated temperature on interspecific competition did not translate into a more apparent dominance of the gobies over the native fish.

Aggression

In single-species treatments, the racer goby was more aggressive than the European bullhead. In contrast, the gudgeon and monkey goby did not differ in level of aggression in the second pair of co-existing species. This finding suggests that aggressive

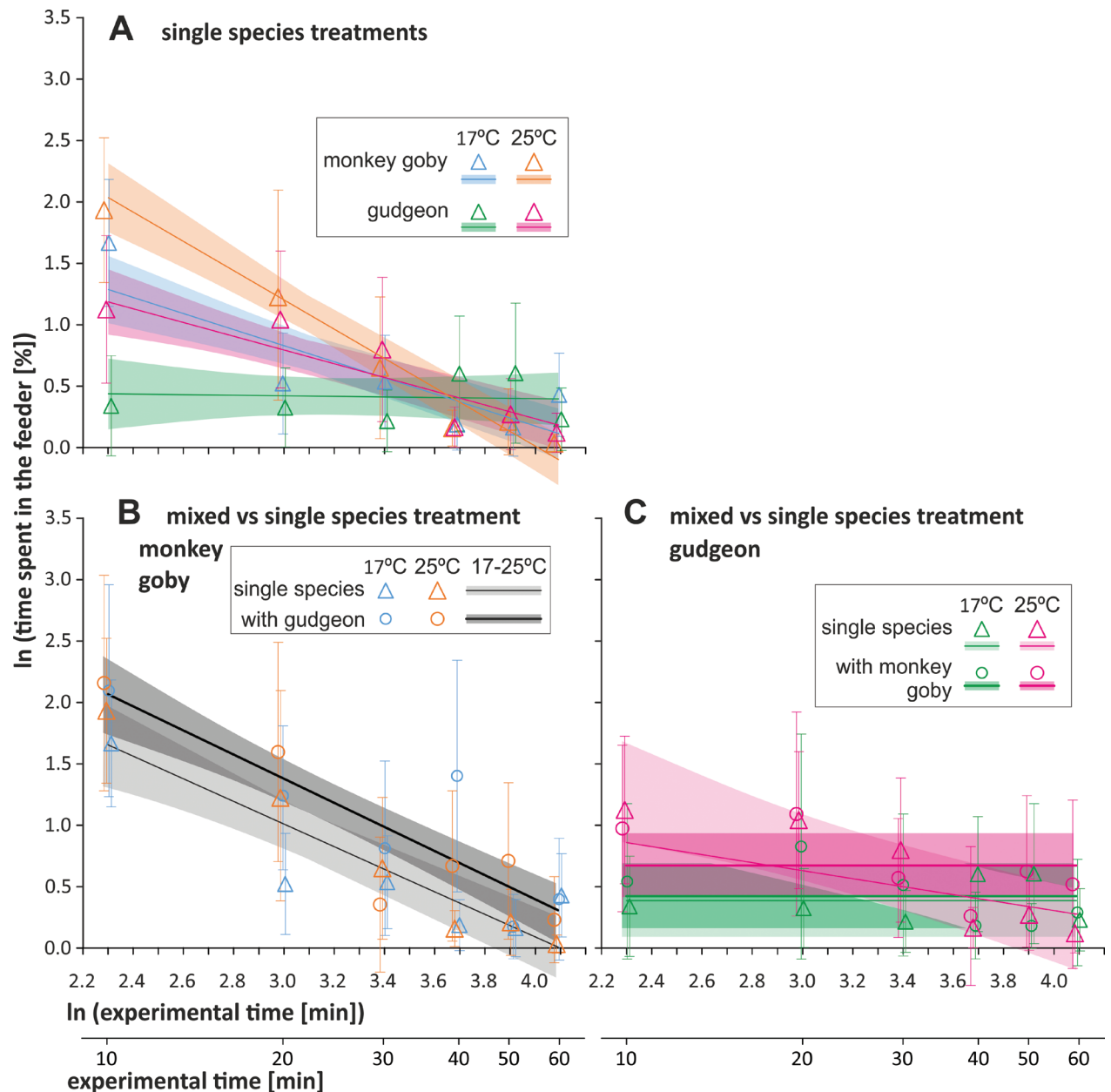


Figure 6. Times spent in the feeder (as percentage of the total exposure time) by the monkey goby and gudgeon kept in separate single-species treatments (A). Panels B, C present comparisons of the behaviour of the monkey goby and gudgeon, respectively, between the single- and mixed-species treatments. Symbols represent raw data (means \pm 95%CI) for each species, temperature and period. Lines are predicted by the models (with 95%CI as shaded areas). Common slopes were predicted for groups of data that did not differ significantly from each other in the models. Horizontal lines indicate non-significant slopes.

behaviour is of primary importance for establishing intra-species dominance in the racer goby, while it is not so in the other species tested. Interestingly, relatively higher aggression was revealed in inter-species interactions in these species in our study (Ladich 1988, 1990, 1997; Hadjiaghai and Ladich 2015; Horvatić et al. 2016, 2021; Fattorini et al. 2023). According to the resource overlap hypothesis (Connell 1983; Britton et al. 2010; Peiman and Robinson 2010), intraspecific aggression is usually stronger than interspecific one because of the greater niche similarity between conspecifics compared to heterospecifics (Kornis et al. 2014). While, in the present work, the native species increased their aggression in the presence of their invasive counterparts. Moreover, the gudgeon was more aggressive towards the monkey goby

than the other way round. These findings are also surprising in the light of the fact that, in general, invasive fish species have been found to display higher levels of aggression than native fish species (Blanchet et al. 2007; Martin et al. 2010; Kakareko et al. 2013), which is considered an important behavioural mechanism determining the competitive superiority of successful invaders (Pintor et al. 2008; Hudina et al. 2014; Silva et al. 2019). Although the opposite situations can be found e.g., in native cichlids: the Kariba tilapia *Oreochromis mortimeri* (Chifamba and Mauru 2017) and the Mexican mojarra *Cichlasoma istlanum* (Archundia and Arce 2019), these are rather rare. In the case of the Ponto-Caspian gobies, earlier laboratory experiments on adult individuals have demonstrated that the higher aggressiveness allowed them to gain an advantage over native species (Kakareko et al. 2013; Jermacz et al. 2015; Grabowska et al. 2016). Nevertheless, relatively high aggression of invasive gobies in those experiments could depend on their larger size (associated with older age and maturity) than that of the gobies tested in the present work (Logue et al. 2011; Funghi et al. 2015; Beltrão et al. 2021; Diatroptov and Opaev 2023), especially during the spawning period (Grabowska et al. 2016). On the contrary, we showed that juvenile specimens of the invasive gobies exhibited lower aggression against their native counterparts (except the gudgeon at 25 °C). It is worth noting that aggression can depend on metabolic traits (Seth et al. 2013; Killen et al. 2014). Species with relatively high standard (resting) metabolism displayed more aggression (Metcalf et al. 1995; Cutts et al. 1998). Indeed, our findings are supported in this respect by the results of Kłosiński et al. (2024), who showed that the native species from the same populations and similar in size to those studied in this work exhibit a higher resting metabolism compared to the invasive gobies. This indicates that juveniles of native gudgeon and European bullhead have a potential to expend energy on activities associated with aggressive behaviour. Although such behaviour, as mentioned earlier, is not displayed by the natives in intra-population interactions, it is activated when confronted with juvenile gobies (less aggressive than older individuals). This suggests that the native fish treat juvenile gobies as weaker opponents than conspecifics when assessing the risk of defeat before deciding to start fighting. Nevertheless, in our study, despite the aggression displayed by the natives, the invasive gobies could reach the feeder more efficiently than their native competitors. Thus, the aggression of the native fish turned out to be ineffective against the alien competitors.

The relationship between temperature and aggression acts can be variable. Elevated temperature can either increase (Wilson et al. 2007; Seebacher et al. 2013) or decrease aggression level (White et al. 2019), or cause no changes in aggression (White et al. 2020). In our study, the gudgeon was less aggressive against the monkey goby at 25 °C than at 17 °C. This inability to maintain the constant level of aggression could be accounted for by the temperature elevated beyond its physiological tolerance (Kłosiński et al. 2024), causing a relatively high energy demand. Thus, the gudgeon might have suppressed costly aggressive acts in favour of reaching the feeder earlier to compensate for metabolic costs associated with elevated temperature (Morgan et al. 2001). From the metabolic point of view, aggressive behaviours are associated with relatively high energetic expenditures (Briffa and Sneddon 2007; Seebacher et al. 2013; Fisher et al. 2021), which are expected to have adverse consequences for fitness in the natives.

Regarding the first pair of fish studied, we found that the aggression shown by the European bullhead and racer goby was independent of temperature. This indicates the potential of the European bullhead to survive in warming waters, assuming they have

access to abundant food resources and meet increased energy needs under such conditions. According to Killen et al. (2013), the greater the metabolic scope, the faster the recovery after the effort, and the lower probability that aggressive behaviour is constrained by maximal metabolic capacity. A higher aerobic scope shown by the European bullhead compared to the racer goby, both at 17 and 25 °C (Kłosiński et al. 2024), can allow it to show a greater flexibility in energy allocation (Maazouzi et al. 2011; Killen et al. 2016). On the other hand, allocating too much energy in aggression can lead to the depletion of energy resources for other life activities, such as anti-predatory defences or foraging (Sneddon et al. 1999; Seebacher et al. 2013; Chifamba and Mauru 2017). Therefore, aggression can be beneficial if food resources are possible to defend (Peiman and Robinson 2010). However, our study suggests this is not the case for juvenile European bullhead facing the racer goby invasion in the wild. This is because, in our experiment, the bullhead aggression was insufficient to effectively defend the food resource against the invasive competitor (see the subchapter below).

Foraging

We posit that the time to enter the feeder and the time spent in the feeder should be considered together. These two behaviours are likely to act together in the same direction to enhance the probability of success in food resource competition. Both gobies tended to reach the feeder before their native counterparts. This was likely to limit foraging of their native competitors and provided the invasives with better access to the richest food resources (directly after the food application), which has also been shown for larger (adult) European bullhead and racer goby (Kakareko et al. 2013). Thus, competition between invasive gobies and their native counterparts is likely to depend on the exploitation of resources by the invaders, successfully reducing the foraging time of the natives (Keiller et al. 2021). This has been demonstrated in our study for the monkey goby-gudgeon pair. Alternatively, even if the native species spent the same time in the feeder as their invasive counterparts, the food could have already been eaten by the gobies, being earlier visitors in the feeder. This has been shown in the racer goby-European bullhead pair in our study.

An elevated temperature delayed the time to enter the feeder by the invasive racer goby, despite the fact that this species originates from a warmer climate than that in its invaded range. Hence, increased temperature may have a disruptive effect not only on natives, but also on invasive species. However, the native species, being less adapted to elevated temperatures, can use even more energy or even limit their foraging (thus causing difficulties with obtaining energy) at 25 °C. Therefore, in the longer term, indirect (exploitation) competition (Vonshak et al. 2012; Newman et al. 2020) may adversely affect native species more than invasives as waters become warmer. It is worth emphasising that of the Ponto-Caspian Gobiidae, the round goby (*Neogobius melanostomus*) has received the greatest attention in terms of successfully competing for food with native fish (Grabowska et al. 2023). Janssen and Jude (2001) proposed that interference competition, rather than exploitation competition, was the primary mechanism for declines in the mottled sculpin populations following the round goby invasion. Less attention has been given to the other goby species from the Ponto-Caspian region, which have expanded to many European inland waters. For the first time, we showed that interspecific competition between juvenile individuals of these gobies and their native counterparts is based on the ability to gain better (faster and longer) access to food resources

rather than on direct aggression. Moreover, the ability to assess their chances and avoid a direct conflict with an opponent allows animals to minimize their energy loss and risk of injuries (Parker and Rubenstein 1981; Moretz 2003; Poulos and McCormick 2014), which is consistent with the non-aggressive (fight-avoiding) behaviour of juvenile invasive gobies in our experiment.

Final remarks

Our study has shown that, regardless of summer temperatures (normal or elevated) that occur in Central European rivers, the juvenile invasive gobies are more effective than their native counterparts in competing for access to limited food resources. This finding broadens the knowledge of the threat posed by the Ponto-Caspian gobies towards native European freshwater fishes (see a review by Grabowska et al. 2023), although it does not support the growing evidence for the negative influence of elevated temperatures on native fish species in competitive interactions with invasive species (Taniguchi et al. 1998; Oyugi et al. 2012; Ramberg-Pihl et al. 2023). In our study, the native fish, although more aggressive, could not effectively compete with the juvenile individuals of the gobies irrespective of temperature. This suggests that, in the wild, the juveniles of the invasive gobies have a competitive advantage over natives, gaining better access to food without the cost of aggression, and will maintain this advantage as waters get warmer. It is worth bearing in mind that the gobies have been proven to outperform the natives in other aspects of global warming. They have lower living costs by keeping a lower resting metabolism at the elevated temperature (Kłosiński et al. 2024) and show a greater physiological tolerance to hypoxia (Kłosiński et al. 2025), which is considered another effect of global warming in fresh waters (Ficke et al. 2007; Jane et al. 2021). Therefore, the future invasion success of the alien gobies owing to efficient food competition may be even enhanced by warming waters, although further studies are needed to confirm this.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

We sampled and used the fish by the permit of the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland, statement no. 30/2022 from 22 June 2022. In addition, the capture and use of the European bullhead, which is protected by law in Poland, was accepted by the Regional Directorate of Environmental Protection in Bydgoszcz, Poland (approval number: WOP.6401.4.52.2022.MO). The procedures conducted within the study met the ASAB/ABS guidelines for the use of animals in research (ASAB Ethical Committee and ABS Animal Care Committee 2019). The housing conditions guaranteed a high level of animal welfare, which was manifested by the overall activity and food intake of the fish throughout the research period. The fish had no external signs of stress or disease. Each specimen was used only once. After the experiments, the European bullhead and gudgeon were released into the wild from which they were caught. In turn, racer goby and monkey goby, as invasive species were euthanized by an overdose of buffered Tricaine Methanesulfonate (MS-222) and disposed of according to the Regulation of the Polish Minister of the Environment from 9 September 2011 (Journal of Laws No. 210, item 1260). Killing was conducted by a qualified, certified person (certificate No. 2355/2015 issued by the Polish Laboratory Animal Science Association).

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Author contributions

PK: Conceptualisation, Resources, Methodology, Investigation, Formal analysis, Data interpretation, Visualisation, Writing-Original draft preparation. JK: Conceptualisation, Resources, Formal analysis, Data interpretation, Visualisation, Writing-Review & Editing. TK: Conceptualisation, Resources, Methodology, Data interpretation, Writing-Review & Editing, Supervision.

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Data availability

The data that support the findings of this study are available from the corresponding author, PK, upon reasonable request.

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Supplementary material 1

Experimental setup

Authors: Piotr Kłosiński, Jarosław Kobak, Tomasz Kakareko

Data type: tif

Explanation note: Experimental setup (all the dimensions are given in mm).

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Link: <https://doi.org/10.3897/neobiota.97.134566.suppl1>

Supplementary material 2

Experimental procedure

Authors: Piotr Kłosiński, Jarosław Kobak, Tomasz Kakareko

Data type: tif

Explanation note: Experimental procedure.

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Link: <https://doi.org/10.3897/neobiota.97.134566.suppl2>

Supplementary material 3

Numbers of replicates and individual size differences in particular experimental treatments

Authors: Piotr Kłosiński, Jarosław Kobak, Tomasz Kakareko

Data type: docx

Explanation note: Numbers of replicates (n) and individual size (total length, TL) differences in particular experimental treatments (pairs of fish in particular species configurations tested at specific temperatures). Individual sizes were compared between the species within each species pair in each experimental treatment using t-tests for dependent samples.

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Link: <https://doi.org/10.3897/neobiota.97.134566.suppl3>

Supplementary material 4

The summary of all the models used in the study

Authors: Piotr Kłosiński, Jarosław Kobak, Tomasz Kakareko

Data type: docx

Explanation note: The summary of all the models used in the study.

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Supplementary material 5

Tests of slope significance for particular levels of categorical factors interacting with exposure time in their effects on the number of aggression events shown by the monkey goby and gudgeon (see Table 2)

Authors: Piotr Kłosiński, Jarosław Kobak, Tomasz Kakareko

Data type: docx

Explanation note: Tests of slope significance for particular levels of categorical factors interacting with exposure time in their effects on the number of aggression events shown by the monkey goby and gudgeon (see Table 2).

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Link: <https://doi.org/10.3897/neobiota.97.134566.suppl5>

Supplementary material 6

Tests of slope significance for particular levels of categorical factors interacting with exposure time in their effects on the time spent in the feeder by the racer goby and European bullhead (see Table 5), as well as by the monkey goby and gudgeon (Table 6)

Authors: Piotr Kłosiński, Jarosław Kobak, Tomasz Kakareko

Data type: docx

Explanation note: Tests of slope significance for particular levels of categorical factors interacting with exposure time in their effects on the time spent in the feeder by the racer goby and European bullhead (see Table 5), as well as by the monkey goby and gudgeon (Table 6).

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Link: <https://doi.org/10.3897/neobiota.97.134566.suppl6>

Research Article

Seeds and vegetative propagules of all selected invasive plants in Europe do not survive industrial composting

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Abstract

Plant material of invasive alien plant species (IAPS) must be appropriately disposed of to prevent unintended spread. The current guidelines in Slovenia and in several other European countries recommend composting only the parts of the plants from which they cannot sprout and reproduce. At the same time, the vegetative propagules and seeds should be incinerated. We tested whether the seeds and vegetative propagules (rhizomes, stolons, tubers, and branches) of 30 selected IAPS survive industrial composting, which is the method of processing collected organic waste and green cut from parks and gardens. Mature seeds and vegetative propagules were packed in metal boxes, which were filled with compost and included in the hygienisation phase of biowaste processing at the Regional Waste Management Centre, RCERO Ljubljana. After the industrial composting for 17 days, seed germination and viability tests were done and compared with a control group of seeds collected from the same plants but not undergoing the composting process. The composted and fresh vegetative propagules were planted in pots with soil, and the number of rooted parts was counted. None of the seeds and the vegetative propagules survived the industrial composting process, and we can conclude that it is safe to dispose of the IAPS like other organic waste or green cut.

Key words: Brown waste, disposal of plant material, germination, green cut, IAPS waste collecting, invasive alien plants, non-native plants, vegetative reproduction, waste management



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Introduction

Invasive alien plant species (IAPS) are causing environmental and ecological problems and are responsible for substantial biodiversity decline in almost all regions of the planet (Vilà et al. 2011; Bellard et al. 2016; Diagne et al. 2021).

In Europe, preventing and minimising the effects of invasive alien species on biodiversity is one of the programmes of the European Commission, resulting in the Invasive Alien Species Regulation (Regulation (EU) 1143/2014; (European Commission 2014). The regulation contains a List of invasive alien species of Union concern, including 41 plant species in 2024. The listed species are subject to restrictions and measures, including restrictions on keeping, importing, selling, breeding, growing and releasing into the environment. However, not all invasive species present in Europe are regulated. For example, the extremely widespread

species *Reynoutria japonica* Houtt. and North American *Solidago* spp., *Phytolacca americana* L., which have been spreading very effectively in the last decades, and the highly allergenic *Ambrosia artemisiifolia* L., are not (yet) included in the EU list. These and several other invasive or potentially invasive alien species and non-native species from alert lists (Essl and Rabitsch 2002; Celesti-Grapow et al. 2009; De Groot et al. 2017; Csiky et al. 2023; EPPO 2024; Nikolić 2024) are at least for now not regulated by the European legislation. The regulation and management of these IAPS is the domain of each European member state.

The economic costs of biological invasions are incredibly high and are still underestimated (Diagne et al. 2021). The prevention and the response in the early phases of plant invasion are much more effective and less expensive comparing interventions in highly invaded areas (Finnoff et al. 2010). However, the early phases are already over in several regions, and in these cases, we have to face the problem of costly plant removal. Usually, the financial and human resources are limited, and in such cases, recognising the high-impact species and prioritising their management is crucial (Blackburn et al. 2014). Actions to remove invasive plants from nature are already a constant practice worldwide.

There is a lot of research on the most effective removal methods for various plant groups, considering the biology and reproduction strategies of different invasive species. Several such scientific publications can be accessed through the websites of organisations that deal with invasive species, e.g., IUCN (<https://iucn.org/our-union/commissions/group/iucn-ssc-invasive-species-specialist-group>), CABI (<https://www.cabi.org/what-we-do/invasive-species/>) or GBIF (<https://www.gbif.org>), national or regional institutions (ministries, agencies and institutes) and different projects dealing with invasive species, as two LIFE projects from Slovenia, Life OrnamentalIAS: <https://zrsvn-varstvonarave.si/blog/projekti/life-ornamentalias/> and Life Artemis: <https://www.tujerodne-vrste.info/project-life-artemis/>).

Despite the worldwide problems with invasive species and numerous studies on their biology, ecology and management, research on disposal methods is still scarce. We found only a few that were dealing with single species (Hassani et al. 2021; Popovic et al. 2021; Wang et al. 2024) or a small group of ecologically connected species (Meier et al. 2014; Strgulc Krajšek et al. 2020). In publications and webpages, primarily written for the general public or different interest groups (for example, Strgulc Krajšek et al. 2016; Dolenc and Papež Kristanc 2020), instructions are based on practical experiences and not on controlled experiments and commonly include the precautionary principle. Robinson et al. (2017), who analysed the websites about the invasive Japanese knotweed (*Reynoutria japonica*), found conflicting information about the potential socio-economic and ecological problems caused by IAPS and contradictory suggestions about the most appropriate management techniques. Burning the collected material is often suggested if the propagules, such as seeds, tubers, etc., are present. Such an approach is safe but expensive. Still, there was no existing research that would analyse the survival rate of invasive alien plant propagules included in regular industrial biowaste processing. Such research is in the interest of scientists who are frequently asked about the effective and safe methods of IAPS disposal and cannot give complete and reliable answers, and in the interest of the institutions dealing with the biowaste, which strive to simplify the collection and processing procedures.

In Slovenia, biodegradable waste including kitchen and garden waste that residents deposit in designated containers (brown waste) from one-fifth of the

households (ca. 470 000 residents) is collected at Ljubljana Regional Waste Management Centre (RCERO Ljubljana) (Sankovič 2017). They also accept yard waste (green cut) from the maintenance of gardens and parks, such as branches, leaves, grass, and water lilies.

The biowaste processing in RCERO Ljubljana has several phases (Fig. 1) (Sankovič 2017). Biowaste from households and yard waste are separately collected in the reception hall. In the first step, all gathered material is ground. The biowaste from households is then sieved, metal particles are removed and then it is transported to the bioreactor, where the fermentation process takes place. After three weeks in the bioreactor, the material is squeezed and transported to the composting tunnels, where it is mixed with previously ground yard waste. The phase of composting in tunnels is 2 to 3 weeks long, and during it, the temperature should be a minimum of 55 °C for at least 4 consecutive days, or above 65 °C for at least 3 consecutive days to meet the standards for hygienisation from the Decree on the treatment of biodegradable waste and the use of compost or digestate (Ministrstvo za kmetijstvo in okolje 2013), which is in accordance to Directive 2008/98/EC of the European Parliament and of the Council (<https://eur-lex.europa.eu/eli/dir/2008/98/oj>). After this phase, the biomass is transported to the maturation hall for four weeks, where it is turned three times a week. During the maturation, the material partially dries so it can be sifted and cleared of plastic particles and other impurities, like stones and bones. The produced compost is supplied to companies that mix the substrates for flowers and gardens. A portion of the compost is used within the municipality for park maintenance. Residents and companies that work on landscaping around houses, green roofs, and similar projects also purchase a smaller portion.

There are several other biowaste processors in Slovenia. One of them processes the biodegradable kitchen waste using anaerobic digestion (biogas plant) only, and the fermentation takes place at lower dry matter content. Aerobic phases do not follow this phase. The digestate is treated as wastewater, and compost is not produced. Other processors have composting facilities and an aerobic process for treating biodegradable waste without an anaerobic component.

The practice of separate collection of plant material of selected IAPS was established in Ljubljana in 2018 as part of the Applause project, in the frame of which researchers studied the possibilities of using IAPS as a raw material for various products (Berden 2019). The selected plant species used in a project were collected separately; additionally, a collection bin for mixed IAPS material was available. All unused material from the containers was sent to incineration to prevent the potential spreading of IAPS. Such practice is safe but expensive.

The main goal of our study was to test the safety of disposing seeds and vegetative propagules of selected invasive plant species that are common across Europe in industrial composting at Ljubljana Regional Waste Management Centre (RCERO Ljubljana). We included the seeds and vegetative propagules in one phase of industrial composting, namely composting with active air ventilation, which took place in the composting tunnels. After the exposure we tested if the propagules survived. Based on the results obtained, we wrote new recommendations for the disposal of biomass of invasive alien plant species (IAPS).

To our knowledge, this is the first experiment where seeds and vegetative propagules of so many invasive species were included in the industrial composting to inspect their viability after the process.

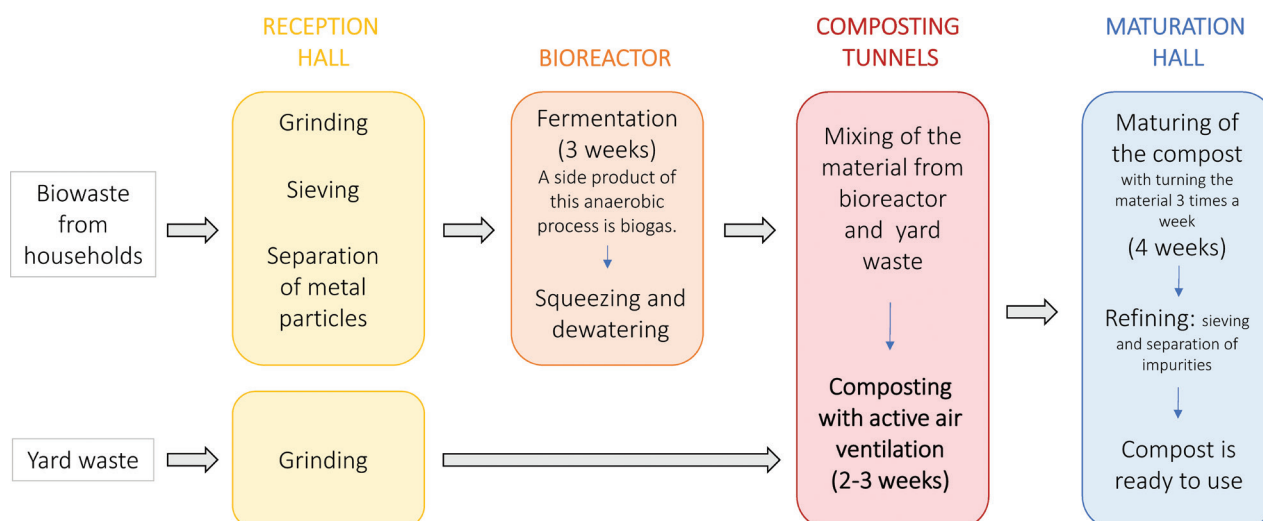


Figure 1. The biowaste processing in RCERO Ljubljana.

Materials and methods

Plant material

The propagules of 30 IAPS, present in Slovenia were tested, 26 as seeds or fruits and 11 as vegetative propagules (Table 1). We included trees and shrubs, vines, perennials and annuals, so we covered a variety of life forms.

The material for the experiment with seeds

We selected 26 invasive alien species already widespread in Europe (Table 1). In September and October 2022, we collected their ripe seeds or fruits, depending on the species' propagule. All material was collected in Slovenia. Additionally, we used the seeds of two commercially available species, *Lactuca sativa* L. and *Raphanus sativus* L. (Royal Seeds, HortuSi srl, Italy, <https://hortus.org>), as a control. Seeds and fruits were dried at room temperature and stored in a dry and dark place at room temperature until they were used.

Before the experiment, we divided plant seeds or fruits of all species into three batches: C—control, T1—first composting, and T2—second composting. The division of propagules into batches was random; however, each batch contained approximately the same number of fruits or seeds. Propagules of control batches (C) were stored in covered but not sealed Petri dishes and placed in a dry and dark place at room temperature. The propagules of test groups T1 and T2 were packed in parcels made of nylon socks. The unique combination of small plastic cubes with letters served as a code for identifying the samples after the composting.

The material for the experiment with vegetative propagules

In March 2024, we collected the vegetative parts that serve as propagules for vegetative reproduction of 11 IAPS (Table 1). We cut the one or two-year-old branches into ca. 15 cm long pieces and washed the underground parts. We divided all the material into three batches: C—control, T1—first composting, and T2—second composting. Each batch contained approximately the same amount of the plant

Table 1. Invasive plant species included in the experiments. Legend to the assigned invasiveness status: EU - the species is on the List of invasive alien species of Union concern (European Commission 2014), EPPO - the species is declared invasive in Europe (EPPO 2024), ISSG - the species is included in the Global Register of Introduced and Invasive Species for at least one European country (GBIF 2024), SLO - the species is on the Slovenian list of IAPS (Strgulc Krajšek et al. 2016).

Species	Invasiveness status	Locality (country, town, coordinates)	Date of collection	Composted part
<i>Acer negundo</i> L.	ISSG, SLO	Slovenia, Ljubljana, 46.048724, 14.603788	1. 10. 2022	Fruits
		Slovenia, Ljubljana, 46.065500, 14.507917	15. 3. 2024	Branches
<i>Ailanthus altissima</i> (Mill.) Swingle	EU, EPPO, ISSG, SLO	Slovenia, Kranj, 46.244287, 14.356486	14. 9. 2022	Fruits
<i>Ambrosia artemisiifolia</i> L.	EPPO, ISSG, SLO	Slovenia, Vikrče, 46.126819, 14.445264	1. 10. 2022	Fruits
<i>Amorpha fruticosa</i> L.	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.041521, 14.514444	22. 9. 2022	Fruits
<i>Asclepias syriaca</i> L.	EU, EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.070490, 14.524841	1. 10. 2022	Seeds
<i>Berberis thunbergii</i> DC.	ISSG, SLO	Slovenia, Kranj, 46.243899, 14.356559	14. 9. 2022	Fruits
<i>Buddleja davidii</i> Franch.	EPPO, ISSG, SLO	Slovenia, Kranj, 46.245129, 14.357795	14. 9. 2022	Fruits
		Slovenia, Ljubljana, 46.065528, 14.472444	14. 3. 2024	Branches
<i>Cornus sericea</i> L.	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.053210, 14.470126	20. 9. 2022	Fruits
			14. 3. 2024	Branches
<i>Echinocystis lobata</i> (Michx.) Torr. & A.Gray	ISSG, SLO	Slovenia, Šmartno, 46.053063, 14.470288	2. 10. 2022	Seeds
<i>Erigeron annuus</i> (L.) Pers.	ISSG, SLO	Slovenia, Rakovica, 46.250422, 14.322822	29. 9. 2022	Fruits
<i>Helianthus tuberosus</i> L.	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.117162, 14.449836	3. 9. 2022	Tubers
<i>Impatiens glandulifera</i> Royle	EU, EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.052692, 14.470819	20. 9. 2022	Seeds
<i>Lonicera maackii</i> (Rupr.) Maxim.	ISSG, SLO	Slovenia, Ljubljana, 46.065806, 14.470667	14. 3. 2024	Branches
<i>Mahonia aquifolium</i> (Pursh) Nutt.	ISSG, SLO	Slovenia, Ljubljana, 46.038189, 14.513496	20. 9. 2022	Fruits
<i>Parthenocissus quinquefolia</i> (L.) Planch.	ISSG, SLO	Slovenia, Kokrica, 46.262577, 14.356993	20. 9. 2022	Fruits
		Slovenia, Ljubljana, 46.061917, 14.468472	14. 3. 2024	Branches
<i>Paulownia tomentosa</i> (Thunb.) Steud.	ISSG, SLO	Slovenia, Ljubljana, 46.262577, 14.356993	2. 10. 2022	Seeds
<i>Physocarpus opulifolius</i> (L.) Maxim.	ISSG, SLO	Slovenia, Kokrica, 46.262171, 14.353872	14. 9. 2022	Seeds
<i>Phytolacca americana</i> L.	ISSG, SLO	Slovenia, Medno, 46.052692, 14.470819	3. 9. 2022	Fruits
<i>Potentilla indica</i> (Andrews) Th. Wolf	ISSG, SLO	Slovenia, Ljubljana, 46.053063, 14.470288	20. 9. 2022	Aggregate fruits
<i>Prunus laurocerasus</i> L.	ISSG, SLO	Slovenia, Ljubljana, 46.040230, 14.512205	20. 9. 2022	Fruits
<i>Reynoutria japonica</i> Houtt.	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.036528, 14.528306	1. 10. 2022	Fruits
		Slovenia, Stanežiče, 46.110611, 14.445639	14. 3. 2024	Rhizomes
<i>Reynoutria × bohémica</i> Chrtek & Chrtková	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.117162, 14.449836	14. 3. 2024	Rhizomes
<i>Rhus typhina</i> L.	ISSG, SLO	Slovenia, Kranj, 46.249424, 14.355008	14. 9. 2022	Fruits
<i>Robinia pseudoacacia</i> L.	ISSG, SLO	Slovenia, Kranj, 46.241528, 14.355463	14. 9. 2022	Seeds
<i>Rosa multiflora</i> Thunb.	ISSG, SLO	Slovenia, Ljubljana, 46.042274, 14.515662	20. 9. 2022	Aggregate fruits
<i>Rudbeckia laciniata</i> L.	ISSG, SLO	Slovenia, Ljubljana, 46.061917, 14.468889	14. 3. 2024	Rhizomes
<i>Solidago canadensis</i> L.	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.117162, 14.449836	2. 10. 2022	Fruits
		Slovenia, Ljubljana, 46.065667, 14.471667	14. 3. 2024	Rhizomes and stolons
<i>Solidago gigantea</i> Aiton	EPPO, ISSG, SLO	Slovenia, Ljubljana, 46.052692, 14.470819	20. 9. 2022	Fruits
		Slovenia, Ljubljana, 46.117162, 14.449836	14. 3. 2024	Rhizomes and stolons
<i>Spiraea japonica</i> L.fil.	ISSG, SLO	Slovenia, Šmarna gora, 46.122526, 14.456736	1. 10. 2022	Seeds
<i>Symphoricarpos albus</i> (L.) K.Koch	ISSG, SLO	Slovenia, Kokrica, 46.262296, 14.356340	17. 10. 2022	Fruits

material. We wrapped the plant material from the test groups (T1 and T2) into garden cover fabric (Vrteks, Tosama) together with a unique combination of small plastic cubes with letters that served as a code and stored the parcels in plastic bags in the fridge at 4 °C until use.

Composting procedure

Two boxes with walls made of perforated steel plates were constructed for the composting experiment. The diameter of the round holes in steel plates was 4 mm. The interior of the box was lined with thin wire mesh with a hole size of 1.5 mm. A metal chain was fixed on the side of each box to mark the box's position in the compost pile. All parcels with plant material from one batch (T1 or T2) were mixed with dry compost and placed into the box. The box was covered with a perforated cover made of the same material as the box.

Boxes with parcels from all batches were included in industrial composting. In the years 2022–2023, we composted seeds, and in 2024 the vegetative propagules. The two boxes with seeds (seed batches T1 and T2) were composted in two different compost tunnels, and the boxes with vegetative propagules (vegetative propagules batches T1 and T2) were in the same compost pile but buried at different positions, as we wanted to avoid the risk of losing some of the material due to drying or decomposition in the time of waiting for the next composting pile to be ready for the start of the composting procedure. All the composting procedures were 17 days long.

The temperature regimes during the composting of seeds (batches T1 and T2) were measured with temperature probes, positioned near each of the boxes. The temperature probe for measuring the temperature regime during the composting of vegetative propagules was positioned between the boxes. In all cases, the temperature was recorded once per hour. The composting procedures in all three compost piles were similar, with some differences in temperature regimes (Fig. 2), but all were in accordance with the valid legislation (Ministrstvo za kmetijstvo in okolje 2013).

The composting piles were ventilated through ground-positioned air nozzles in two ways: with air pressure and suction to regulate the temperature in compost piles. For detailed information, see Suppl. materials 1, 2.

Germination and seed viability tests

After composting, the seeds were spread to open Petri dishes and dried at room temperature for a few days. By drying the seeds, we imitated the compost maturation phase in the maturation hall, during which the compost matures and dries (Fig. 1). Afterwards, we covered the Petri dishes and placed all the seeds, including the control batch, which was stored at room temperature until then, in the refrigerator at 4 °C for 2 months to break the potential dormancy of seeds.

Before the viability tests, we isolated seeds from fruits or their decomposed parts. All seeds were also washed with running tap water. Some seeds from the series T1 and T2 have partly decomposed or damaged during the composting. In those cases, visually, the complete seeds were selected for germination experiments. We scarified the seeds of *Robinia pseudoacacia* with a scalpel and crushed the *Prunus laurocerasus* pit before including them in the germination experiment.

The germination of most of the seeds was tested in Petri dishes on filter paper watered with tap water. The petri dishes were kept in growing chambers at 24 °C, 12 hours of light per day and 50% air humidity. The *Ambrosia artemisiifolia* seeds

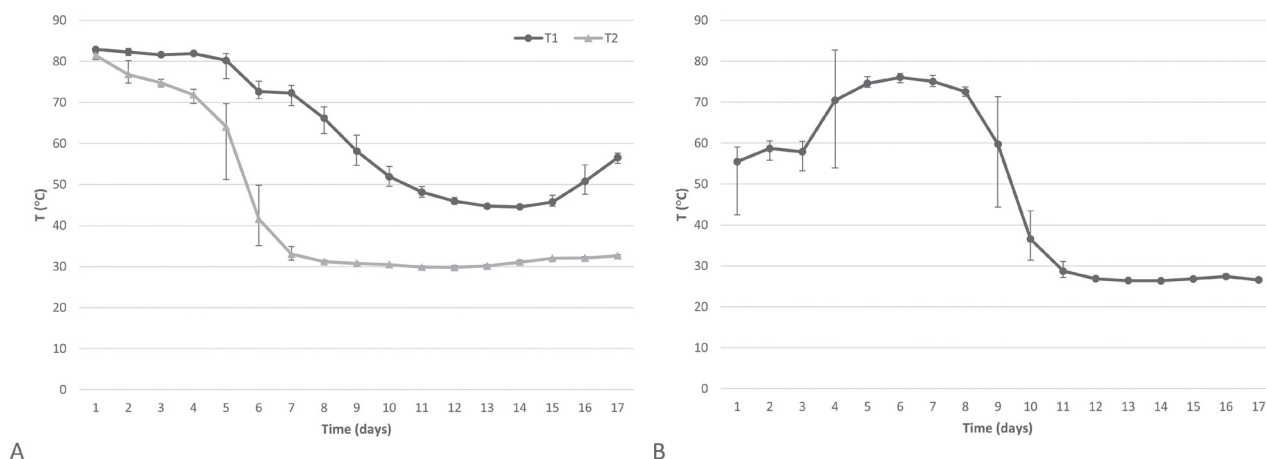


Figure 2. Average day temperature in the compost pile beside the boxes with seeds of batch T1 (black line) and T2 (grey line) (A) and during the composting of the vegetative propagules (B). Vertical bars show minimum and maximum temperatures.

were moved from the growing chamber to the fridge (4 °C) every night. In some cases, we used germination trays filled with soil. The planters were covered with transparent plastic covers and kept in the same conditions as Petri dishes. The trays were watered with tap water twice a week when we observed that the soil started to dry. This method was also used when the germination tests in Petri dishes were unsuccessful. The information about the length and the types of germination experiments is presented in Tables 2, 3.

In cases when control seeds did not germinate, we tested the potential viability of the seeds using the Tetrazolium viability test (Table 3) (Cottrell 1947). We randomly selected the subsample of seeds that did not germinate, cut the seeds in half, placed cut seeds in the holes of microplates, then covered them with a few drops of 1% triphenyl tetrazolium chloride (TTC) and kept them in the dark at room temperature for 20 h. The embryos that were coloured dark red after the incubation were counted as viable.

Testing the viability of propagules for vegetative reproduction

The vegetative propagules were planted in marked box planters filled with commercially available universal non-acidic soil for plants (Substral, Slovenia).

The vegetative propagules of the control group were planted the same day as the material was collected. Rhizomes and tubers were put in the container on the soil and covered with an approximately 2 cm thick layer of soil. We stuck the ca. 5 cm long lower part of the cut branches into the soil. The upper parts stayed above ground. The box planters were left uncovered in an indoor place with natural light at 22 °C in Ljubljana for 2 months (middle of March to middle of May). The light regime was natural, twice a week watering with tap water to prevent the soil from drying out.

The composted material was planted in the same way as the control, but one month later, so the experiment took place from the middle of April to the middle of June. The conditions were the same as for the control, but the light period was longer because the box planters were in the room with natural light. Each box planter contained one species's material and from one treatment only. The quantities of the planted vegetative propagules are given in Table 4.

Table 2. Germination of seeds of invasive plant species and two commercially available crop species before and after composting. Control seeds are seeds that were not composted, T1 and T2 represent two batches of composted seeds.

Species	Germination test (S-soil, P-Petri dish)	Duration of the experiment [days]	Number of seeds per test (replicates)	Germination [%]		
				Control	T1	T2
<i>Acer negundo</i>	S	14	25 (5)	68	0	0
<i>Ailanthus altissima</i>	S	57	25 (5)	0	0	0
<i>Ambrosia artemisiifolia</i>	P	21	100 (4)	34	0	0
<i>Amorpha fruticosa</i>	P	14	25 (5)	44	0	0
<i>Asclepias syriaca</i>	P	28	100 (4)	0	0	0
<i>Berberis thunbergii</i>	P	14	100 (4)	0	0	0
	S	42	100 (4)	0	0	0
<i>Buddleja davidii</i> *	S	21	not counted (4)	22*	0	0
<i>Cornus sericea</i>	P	14	100 (4)	0	0	0
	S	42	100 (4)	0	0	0
<i>Echinocystis lobata</i>	P	35	10(2)	0	0	0
<i>Erigeron annuus</i>	P	7	100 (4)	90	0	0
<i>Impatiens glandulifera</i>	P	14	50 (2)	6	0	0
<i>Mahonia aquifolium</i>	P	28	100 (4)	0	0	0
<i>Parthenocissus quinquefolia</i>	P	28	100 (4)	0	0	0
<i>Prunus laurocerasus</i>	P	56	100 (4)	0	0	0
<i>Paulownia tomentosa</i>	P	12	100 (4)	87	0	0
<i>Physocarpus opulifolius</i>	P	21	100 (4)	17	0	0
<i>Phytolacca americana</i>	P	56	50 (2)	46	0	0
<i>Potentilla indica</i>	P	28	100 (4)	36	0	0
<i>Reynoutria japonica</i>	P	21	100 (4)	0	0	0
<i>Rhus typhina</i>	P	35	100 (4)	0	0	0
<i>Robinia pseudoacacia</i>	P	7	25 (5)	88	0	0
<i>Rosa multiflora</i>	P	35	100 (4)	0	0	0
<i>Solidago canadensis</i>	P	15	100 (4)	21	0	0
<i>Solidago gigantea</i>	P	20	100 (4)	48	0	0
<i>Spiraea japonica</i>	P	12	100 (4)	97	0	0
<i>Symphoricarpos albus</i>	P	14	100 (4)	0	0	0
	S	42	100 (4)	0	0	0
<i>Lactuca sativa</i>	P	6	50 (2)	96	0	0
<i>Raphanus sativus</i>	P	6	50 (2)	84	0	0

*In the case of *Buddleja davidii*, the number of seeds was not counted because they were not recognisable after the composting. Therefore, the unknown number of seeds was sown, and the number marked with a star symbol represents the number of seedlings, not the percentage of germinated seeds.

Table 3. Metabolic activity of seeds of selected invasive plant species before and after composting, obtained from the Tetrazolium test. Control seeds are seeds that were not composted, T1 and T2 represent two batches of composted seeds.

Species	Number of tested seeds	Viable seeds [%]		
		Control	T1	T2
<i>Ailanthus altissima</i>	24	25	0	0
<i>Asclepias syriaca</i>	24	75	0	0
<i>Berberis thunbergii</i>	24	54	0	0
<i>Cornus sericea</i>	24	63	0	0
<i>Echinocystis lobata</i>	10	100	0	0
<i>Reynoutria japonica</i>	20	0	0	0
<i>Mahonia aquifolium</i>	24	92	0	0
<i>Parthenocissus quinquefolia</i>	24	100	0	0
<i>Prunus laurocerasus</i>	24	21	0	0
<i>Rhus typhina</i>	24	0	0	0
<i>Rosa multiflora</i>	24	25	0	0
<i>Symphoricarpos albus</i>	24	8	0	0

Results

Germination and seed viability

The collected seeds of all species except *Reynoutria japonica* and *Rhus typhina* were viable according to the germination experiments or the Tetrazolium viability tests. However, none of the seeds from series T1 and T2 survived the composting. The results of the tests are shown in Tables 2, 3.

The viability of propagules for vegetative reproduction

All the vegetative propagules of the selected invasive plant species, except the branches of *Cornus sericea*, were viable. At least some of the vegetative propagules used in the control experiment developed roots and green shoots (Table 4). The industrial composting destroyed all the viable parts of the plant material and none of the planted composted fragments developed roots or shoots (Table 4).

Table 4. Vegetative propagation of selected invasive plant species before and after industrial composting. Control are the propagules that were not composted, T1 and T2 represent two batches of composted vegetative propagules. Legend: T-tuber, R-rhizome, B-branch.

Species	Propagule	Number of propagules	Number of viable propagules with roots (total number of shoots)		
			Control	T1	T2
<i>Acer negundo</i>	B	8	3(3)	0	0
<i>Buddleja davidii</i>	B	18	4(6)	0	0
<i>Cornus sericea</i>	B	14	0(0)	0	0
<i>Helianthus tuberosus</i>	T	9	9(15)	0	0
<i>Lonicera maackii</i>	B	7	6(6)	0	0
<i>Parthenocissus quinquefolia</i>	B	14	6(6)	0	0
<i>Reynoutria × bohemica</i>	R	7	5(15)	0	0
<i>Reynoutria japonica</i>	R	5	5(7)	0	0
<i>Rudbeckia laciniata</i>	R	6	6(22)	0	0
<i>Solidago canadensis</i>	R	7	7(22)	0	0
<i>Solidago gigantea</i>	R	10	10(15)	0	0

Discussion

Of the seeds or vegetative propagules of 30 different plant species that are invasive in Slovenia and Europe, none survived the industrial composting in composting tunnels at RCERO Ljubljana. The propagules of IAPS may occur in two sources of biowaste commonly collected in Ljubljana: brown waste, which is collected in households, and yard waste from the maintenance of gardens and parks. Households usually dispose of smaller amounts of plant biomass removed from gardens and put them in bins for organic waste. However, the material from parks and public areas and the collected material of IAPS during public actions are usually transported directly to the collection centres as the green cut.

The phase of composting in tunnels is common to both sources of biowaste (Fig. 1), and this is the reason that we decided to include the propagules in this phase only. In this phase, the temperatures rise above 70 °C for a few days (Fig. 2). For optimal composting long periods of high temperatures must be avoided to allow the development of eumycetes and actinomycetes, which are the main decomposers of the long-chain polymers, cellulose and lignin (de Bertoldi et al. 1983). The most effective way to control the temperature is forced pressure ventilation and turning the composting material (de Bertoldi et al. 1983).

The biowaste from households in RCERO Ljubljana also goes through the anaerobic fermentation phase, where the temperature is $55(\pm 1)^{\circ}\text{C}$, and this phase can additionally reduce the chance of survival of seeds and vegetative propagules. However, in the commercial mesophile bioreactors, the temperatures are lower. In the experiment made by Hahn et al. (2023), where T did not exceed 42°C , hard-seed species, such as *Malva sylvestris* L., survived the procedure, but the seeds with softer testa, such as *Chenopodium album* L., were completely inactivated by the treatments. Johansen et al. (2013) exposed seeds to anaerobic digestion at higher temperatures (55°C) and complete mortality of all 7 tested plant weeds after two days. They report that the temperature seemed to be the major cause of damage, as the same species germinated after the anaerobic digestion at 37°C . The survival of seeds after the anaerobic digestion at 37°C was reported also by Westerman et al. (2012).

The reason that seeds and vegetative propagules did not survive the process of hygienisation must be the combination of high temperatures, humid environment, high concentration of ammonia, pathogen infestations, water-soluble organic phytotoxins, and microorganisms in the substrate, as was already reported by Hassani et al. (2021), who did the composting experiment of *Lupinus polyphyllus* Lindl. seeds, and several others, for example, Zaller (2007), Eghball and Lesoing (2000) and Johansen et al. (2013). High temperature is most probably not enough to kill the seeds of all species. Seeds of different plant species have different tolerance to high temperatures. So, several of the tested IAPS may have seeds sensitive to temperatures higher than 55°C . The extreme tolerance was reported by Daws et al. (2007), who discovered that seeds of some desert succulents survive exposure to 103°C for 17 hours. The seeds of the black locust (*Robinia pseudoacacia*), which we tested in our experiment, can survive the increased temperatures during the fire. Popovic et al. (2021) exposed the seeds of black locust to 100°C for 2.5 and 5 minutes and tested their germination. It was significantly lower compared to the control seeds, but several seeds survived the exposure. In our experiment, black locust seeds did not survive the composting process despite lower temperatures during the process (Fig. 2). Even the lower temperature, at least 57.2°C , was enough to destroy the propagules of aquatic invasive plant species *Arundo donax* L., *Hydrilla verticillata* (L.f.) Royle, *Eichhornia crassipes* (Mart.) Solms, and *Pistia stratiotes* L. from the Rio Grande River, during the large-scale composting experiment (Meier et al. 2014). In this experiment, the whole plants containing vegetative propagules and seeds were composed, and the result was a valuable compost product without the viable propagules of IAPS.

In our experiment, the seed viability of the control group and both batches of composted seeds was tested by the combination of a germination test and a test of metabolic activity by Tetrazolium staining, which was used when the control seeds did not germinate (most probably because of dormancy or unsuitable germination conditions). None of the methods have shown that the seeds would survive composting. The control seeds of all species, except *Rhus typhina* and *Reynoutria japonica*, were viable. We found that all the seeds of *Rhus typhina* were empty. We checked the seeds from another locality, and they were empty, too. In Slovenia, we have not yet observed the propagation of this species by seeds, but vegetative propagation with stolons is very common. The ripe fruits of *Reynoutria japonica* were collected at the site, where we had already collected the viable seeds for other experiments. Hence, the result that the seeds were not viable was unexpected. Regarding the results of other tested IAPS, we do not expect that viable *Reynoutria*

japonica seeds will survive composting. Similarly, the branches of *Cornus sericea* of the control group did not grow roots. *Cornus sericea* is a species that vegetatively reproduces mainly by ground layering, where the stem is bent down and partly buried in the soil while still attached to the parent plant (Bačič et al. 2015). However, cut branches also can root and serve as vegetative propagules (Strgulc Krajšek et al. 2020). Any other branches or rhizomes that we used in the experiment did not survive the composting. The vital parts of all branches, tubers and rhizomes were almost completely decomposed, only the wooden parts and bark remained.

Based on the fact that 100% of the tested species were inactivated during composting, we believe that we can state with high probability that the industrial composting of IAPS is safe in terms of preventing the spread of IAPS into nature by using the obtained compost, when the composting process meets the requirements of “Decree on the treatment of biodegradable waste and the use of compost or digestate” (Ministrstvo za kmetijstvo in okolje 2013) and Directive 2008/98/EC of the European Parliament and of the Council (<https://eur-lex.europa.eu/eli/dir/2008/98/oj>).

New recommendations for the disposal of IAPS biomass can be summarised in the following two points:

1. Plant material without seeds or vegetative propagules can be composted in garden compost heaps or disposed of as other household organic waste or green waste from parks and gardens. Disposing of any garden plant material in natural environments, such as riverbanks, forests, or forest edges, must be prohibited.
2. Plant material containing seeds (even if not fully ripe) or vegetative propagules (such as rhizomes, bulbs, tubers, and branches that can regrow) should be disposed of as household organic waste (brown waste) or yard waste from parks and gardens intended for industrial composting. When composting process complies with the standards set by the “Decree on the treatment of biodegradable waste and the use of compost or digestate” (Ministrstvo za kmetijstvo in okolje 2013) and Directive 2008/98/EC of the European Parliament and of the Council (<https://eur-lex.europa.eu/eli/dir/2008/98/oj>), there is no risk of spreading IAPS through the use of the produced compost.

Conclusion

We used the seeds or vegetative propagules of 30 different IAPS that are invasive in Slovenia. We included trees and shrubs, vines, perennials and annuals, so we covered a variety of life forms. As there were no observed viable propagules after the composting, we proposed the new management recommendation that waste from IAPS can be composted and does not have to be incinerated. This less complicated protocol may simplify and reduce the costs of IAPS disposal and can have another positive effect, namely that more residents will choose to dispose of plant material of IAPS in bins for organic waste or bring it to landfills, and less of the IAPS will end up in compost heaps in the wild, that is still a common practice in Slovenia (Šipek and Šajna 2020).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: SSK. Formal analysis: NG, ST. Funding acquisition: SSK. Investigation: NG, SSK, ST, MR. Methodology: MR, SSK, ST. Resources: SSK. Supervision: SSK. Validation: SSK. Visualization: SSK. Writing - original draft: ST. Writing - review and editing: SSK, NG, MR.

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Data availability

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

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Supplementary material 1

The information about the composting procedures

Authors: Marjeta Resnik

Data type: xlsx

Explanation note: The file consists of three spreadsheets, each containing one table with the parameters measured during the composting procedures. The parameters are: retention time, temperature, duration of ventilation with air pressure, and duration of ventilation with air suction.

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Supplementary material 2

Daily duration of the two types of ventilation during composting of seeds (TS-1 and TS-2) and vegetative propagules (T-V)

Authors: Marjeta Resnik






Data type: docx

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Research Article

Alien species of *Ipomoea* in Greece, Türkiye and Iran: distribution, impacts and management

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Abstract

New introductions of alien *Ipomoea* species and their negative impacts have increased in Greece, Türkiye, and Iran. However, little is known about their current status, distribution, impacts, and management. Here, we provide a detailed overview of *Ipomoea* species in these countries and discuss the current and proposed management options for restoring invaded plant communities. We report on four alien *Ipomoea* species in Greece (three naturalized and one casual), 10 in Türkiye (eight naturalized and two casual), and 11 in Iran (eight naturalized of which two are invasive and three casual). Their most significant negative impact was detected in agricultural areas, especially in spring crops like soybean, cotton, and maize, with *I. hederacea*, *I. purpurea*, and *I. triloba* being the most troublesome weeds. Native plants are mainly threatened by *I. indica*, *I. leucantha*, and *I. triloba*. The management of *Ipomoea* species differs according to the habitats invaded; in agricultural areas, the management is more dependent on the host crop and the available registered herbicides, whereas in areas with natural habitats, other management options such as mechanical and biological measures are more appropriate. The information from this work will be useful for the early detection of *Ipomoea* species in countries neighboring the already invaded ones.

Key words: Biological invasion, casual plant, herbicides, management, naturalized plant



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Introduction

Invasive alien species (IAS) are considered one of the greatest threats to native ecosystems and biodiversity (Pyšek et al. 2020a; IPBES 2023) and have received increasing attention from scientists and policy-makers in recent decades due to their detrimental ecological impact (Hejda et al. 2009; Pyšek et al. 2012; Blackburn et al. 2014; Kumschick et al. 2015; Rumlerová et al. 2016) and economic cost (Novoa et al. 2021; Diagne et al. 2022; Tarkan et al. 2024). Plants are the most represented group of alien organisms and, similar to other groups, their numbers

in invaded regions are increasing globally, as are the rates of new introductions (van Kleunen et al. 2015; Pyšek et al. 2017; Seebens et al. 2017, 2019).

The taxonomic distribution of plant families and genera in the global naturalized flora is highly heterogeneous, with at least one naturalized species present in 287 families and 2984 genera (van Kleunen et al. 2019). One of the genera with the highest representation of naturalized aliens worldwide is *Ipomoea* from the family Convolvulaceae (Pyšek et al. 2017). The etymology of the name *Ipomoea* comes from the Greek words “*ips*” meaning “worm” and “*homoios*” meaning “similar to”, reflecting the wormlike twining habit of the genus (Rojas-Sandoval and Acevedo-Rodríguez 2014). Pyšek et al. (2017), in their overview of global naturalized flora, reported 55 *Ipomoea* species (i.e., 11.8% of all taxa in the genus) and 1348 region × species records; they considered 47 taxa as naturalized on mainland worldwide and 42 on islands.

This global picture of the invasion by *Ipomoea* taxa can be enhanced by using regional data from different parts of the world. For example, in Congo, there are 11 alien/naturalized *Ipomoea* species among 20 members of Convolvulaceae (Bordbar and Meerts 2022); in Oman, the corresponding proportion was four out of five (Patzelt et al. 2022); in Pakistan, nine out of 14 species (Jehangir et al. 2024) and in India, 12 *Ipomoea* species were reported among the 19 Convolvulaceae species (Inderjit et al. 2018). Approximately 20–25 very widespread naturalized and invasive *Ipomoea* species have been reported in almost all tropical countries, in both the Old and New World (Wood and Scotland 2017; Wood et al. 2020). However, *Ipomoea* species have been widely introduced for ornamental purposes (GBIF 2024; Royal Botanic Gardens Kew 2024) or unintentionally through contaminated seeds of crops such as soybean, cotton, or oilseeds (CABI 2024). They have been reported as invasive in many countries, such as Japan (Mito and Uesugi 2004), India (Chandra 2012), China (Liu et al. 2016; Hao and Ma 2022), Hawaii (PIER [Pacific Islands Ecosystems at Risk] 2017), and South Africa (Pyšek et al. 2020b). Taking into account their widespread introductions and strong invasion potential, more information on their taxonomy and geographical distribution is essential for selecting appropriate management options. This knowledge will help to prioritize the species that are problematic in a particular area and establish management programs accordingly (Pyšek et al. 2020a, b). Thus, preventive measures can be taken, which is one of the first priorities for the management of potential invasive taxa that may cause problems in an area. In addition, gaps in their management can be identified in advance and thus management programs can be developed against possible risks (Pyšek et al. 2020a; García-Díaz et al. 2022).

Ipomoea taxa are annual or perennial climbing herbs, shrubs or even small trees; the genus comprises approximately 600 taxa (Mabberley 2017). Some perennial taxa have tubers on their roots and exhibit strong phenological patterns, such as dormancy during cold and dry winters (Muñoz Rodríguez et al. 2019; Wood et al. 2020). Species of the genus *Ipomoea* have a C3 photosynthetic pathway and are thus likely to benefit from the rising atmospheric CO₂ due to climate change (Velumani et al. 2017). For instance, it has been reported that a higher frequency of heat disturbance under global warming will increase the invasiveness of *I. cairica* while suppressing the growth of the native vine *Paederia scandens* in southern China (Chen et al. 2023). The negative impacts of *Ipomoea* taxa are related to the mechanisms that make them strong competitors, such as phototropism, circumnutating, and shade avoidance responses (Price and Wilcut 2007; Paul and Yavitt 2011; Pagnoncelli et al. 2017; Pazzini et al. 2022; Asami et al. 2023), as well as having a fast growth rate and rapid and easy dispersal by seeds and stem fragments (Norsworthy

and Oliver 2002; Kati and Giannopolitis 2006; Moura and Morim 2015; Giraldehi et al. 2019; Helman et al. 2020; Onen et al. 2023). In addition, some species are allelopathic or may host plant pathogens (Shen et al. 2019; Cabral et al. 2023), increasing their negative impact on the local indigenous flora of invaded areas.

Greece, Türkiye and Iran, which form a longitudinal gradient stretching from from 21° W, have suitable biological and ecological characteristics for the establishment of *Ipomoea* species, leading to a rapid increase in the number of reported new records of these taxa. *Ipomoea* species invade a wide range of habitats across both natural and agricultural lands. For instance, soybean fields in USA, Iran and Japan (Howe and Oliver 1987; Sohrabi et al. 2017; Asami et al. 2023), cotton and corn fields in Greece and Türkiye (Giannopolitis and Papachristos 1997; Yazlık et al. 2018), coastal areas in China (Liu et al. 2016) and temperate rainforest in Australia (Bernich et al. 2024) have been severely affected by these species.

Appropriate management of invasive or potentially invasive alien plants depends on detailed information on their pathways of introduction, status, impacts, and distribution (Pyšek et al. 2004; Pergl et al. 2017; Ricciardi et al. 2017; Potgieter et al. 2022). Concerted management actions are needed to prevent their further spread and to minimize the harmful impacts, especially in countries with a wide range of natural habitats and rich native floras (Arianoutsou et al. 2010; Uludağ et al. 2017). The aim of the current paper is to provide a detailed overview of *Ipomoea* species in the affected countries, including current distribution status, and detected impacts. We also provide an overview of management options, current and proposed, to restore *Ipomoea*-invaded plant communities.

Materials and methods

Study area

Greece is a southeastern European country occupying the southern part of the Balkan Peninsula, with a latitude of 39.0742°N and a longitude of 21.8243°E. It has land borders with Albania, North Macedonia, Bulgaria to the north, and Türkiye to the east, and is surrounded by the Aegean Sea (east), the Cretan and the Libyan Seas (south), and the Ionian Sea (west). It has a total area of 131,957 km², of which about 83% is mainland. It has a coastline of 13,676 km, the longest in Europe, and about 6000 islands, of which 227 are inhabited. Almost 80% of Greece is mountainous, with Mount Olimbos (Olympus) being the highest mountain (2,918 m a.s.l.). The Macedonian (northern) and Thessalian (central) plains are the largest in the country and are mainly used for agriculture, horticulture, and forestry. The climate of Greece is predominantly Mediterranean, with mild and rainy winters, warm and dry summers and, generally, long periods of sunshine throughout most of the year. Greece has one of the richest vascular floras in Europe, with 5,959 species and 2,013 sub-species (native and naturalized), representing 6,846 taxa (Dimopoulos et al. 2023).

Türkiye is a large peninsular country between 36°42' north latitude and 26°45' east longitude, with a total land area of 783,562 km². The southern border is defined by the island of Cyprus and the Mediterranean Sea, the western border by the Aegean Sea, and the northern border by the Black Sea. Most of its land mass is in Anatolia, with a small part in Thrace, the south-eastern extension of the Balkan Peninsula. Geographically, Türkiye lies on the border between Europe and Asia. The Sea of Marmara and the Straits of Istanbul and Çanakkale separate Anatolia from

Thrace, effectively dividing Asia from Europe. Due to Türkiye's location as a transition point between Asia and Europe and the three distinct phytogeographic regions it encompasses (Mediterranean, Iran-Turanian, European-Siberian), the country has a rich flora, with more than 12,000 plant taxa. The majority are native taxa, and 31% of the native taxa are endemic (Güner et al. 2000; Uludağ et al. 2017).

Iran, with an area of 1,648,195 km², is located in the arid belt of the eastern hemisphere, in western Asia, between the northern latitudes of 25–45° and the eastern longitudes of 44–63°. It is bordered to the north by the Caspian Sea and to the south by the Persian Gulf and the Sea of Oman. Two high mountain ranges, the Alborz in the north and the Zagros in the west, play a vital role in preventing the Mediterranean and the Caspian Sea winds near the central plateau in Iran. The vascular flora of Iran consists of nearly 8,660 species, of which 2,760 are endemic (Mozaffarian 2024).

Data collection

We used local and regional journals and book chapters, supplemented by Google searches to locate grey literature publications, such as country reports and proceedings articles not included in the scholarly databases. The search included literature related to alien *Ipomoea*, using the following terms: “*Ipomoea* species” or “alien *Ipomoea*” or “new records of *Ipomoea* species” plus country name (Greece, Türkiye and Iran). Our final list comprised 35 documents, including 10 journal articles, two technical reports, and three other documents for Iran; 16 journal articles and three grey citations (three master theses – Arslan 2022; Doğru 2023; Saruhan 2024) for Türkiye; nine journal articles and two online databases for Greece. Information on *Ipomoea* species and their current status, actual impact, and management in all three countries was searched using combinations of keywords such as: “*Ipomoea* species invasion”, “invader”, “alien species”, “exotic species”, “*Ipomoea* management/control”, “weed management in maize/cotton/soybean” and “country name” either as topic or title from local studies and literature review. In addition, some information was obtained from field observations made during plant growth (end of May to October for Iran, May to November in Türkiye) during 2019–2024. Other data on general aspects of the studied species were obtained through Google searches from different databases and research articles.

The stage that the alien *Ipomoea* species under study reached in the naturalization/invasion process (Richardson et al. 2000) was identified for each country: (i) casual alien, (ii) naturalized species (synonym: established species), and (iii) invasive species (Richardson et al. 2000; Blackburn et al. 2011). We also assigned each species to the most commonly invaded habitat types (Suppl. material 1) in the three countries, using the classification of the SynHab project (www.synhab.com; Hejda et al. 2015; Pyšek et al. 2022; Dawson et al. 2025). The following habitat types were distinguished to classify species' habitat preferences: 1. Forest, 2. Open forest, 3. Scrub, 4. Grassland (divided into 4a. Natural grassland, 4b. Human-maintained grassland), 5. Sandy, 6. Rocky, 7. Dryland, 8. Saline, 9. Riparian, 10. Wetland, 11. Aquatic, 12. Artificial (divided into 12a. Ruderal/urban habitats, 12b. Agricultural habitats).

We used the WGS84 geographic coordinate system with an EPSG code of 4326 to map the distribution of the 14 identified species. The map was created at a scale 1:10,000,000 and utilized the image was generated using QGIS and Esri World Data 2021. Geographical coordinates of the detected species were taken from GPS and regional databases (Flora Hellenica database, personal communication with Arne Strid).

Distribution of *Ipomoea* taxa in Greece, Türkiye and Iran

Ipomoea taxa in Greece

Four alien species of *Ipomoea* have been recorded in Greece: *Ipomoea purpurea*, *I. hederacea*, and *I. indica* are naturalized, and *I. batatas* is casual (Fig. 1, Table 1). *Ipomoea purpurea*, which was introduced as an ornamental and has escaped and successfully established in various ecosystems, is the most widespread (Strid 2024). *Ipomoea hederacea*, locally known as ‘agriofasoulia’ (wild bean), is the most recently introduced species of *Ipomoea* in Greece, first reported in the early 1990s in the Preveza Prefecture (western Greece). It was probably introduced as a seed contaminant and caused major problems in irrigated summer crops, particularly in maize and cotton (Giannopolitis and Papachristos 1997; Drolia 2004; Giannopolitis et al. 2004; Kati and Giannopolitis 2004, 2006; Anagnou-Veroniki et al. 2008). Later studies reported that this weed has spread to several other cultivated areas in western Greece and in Thessaly (central Greece), infesting cotton-growing areas of in the Prefecture of Karditsa (Kati and Giannopolitis 2017). *Ipomoea batatas* was cultivated in the country before 1962; its introduction probably occurred earlier as in the case of many other crops (Valíček et al. 2002; Hobhouse et al. 2004; www.fao.org/faostat). It sometimes reproduces in the wild through underground propagules.

Table 1. The *Ipomoea* species in Greece, Türkiye and Iran, their invasion characteristics and introduction pathways.

	Species	Invasion status	Year of introduction	*Habitat type codes	Introduction pathway
Greece	<i>Ipomoea batatas</i> (L.) Lam.	Casual	>1962	12b	Release
	<i>Ipomoea hederacea</i> Jacq.	Naturalized	1994	12a, 12b	Contaminant
	<i>Ipomoea indica</i> (Burm.) Merr.	Naturalized	1972	12a, 12b	Release
	<i>Ipomoea purpurea</i> (L.) Roth	Naturalized	1986	12a, 12b	Contaminant
Türkiye	<i>Ipomoea batatas</i> (L.) Lam.	Casual	1900	12a, 12b	Release
	<i>Ipomoea cocinea</i> L.	Naturalized	2016	12a, 12b	Escape
	<i>Ipomoea hederacea</i> Jacq.	Naturalized	1999	12a, 12b	Escape / Contaminant
	<i>Ipomoeahederifolia</i> L.	Naturalized	2016	12b	Escape / Contaminant
	<i>Ipomoea indica</i> (Burm.) Merr.	Naturalized	2018	12a	Escape / Release
	<i>Ipomoea lobata</i> (Cerv.) Thell.	Casual	2023	12a	Cultivated in a Botanical Garden
	<i>Ipomoea nil</i> (L.) Roth	Naturalized	Unknown	Data Deficient	Data Deficient
	<i>Ipomoea purpurea</i> (L.) Roth	Naturalized	1918	12a, 12b	Escape / Contaminant
	<i>Ipomoea tricolor</i> Cav.	Naturalized	2016	12a, 12b	Escape / Contaminant
	<i>Ipomoea triloba</i> L.	Naturalized	2000	4a, 9, 12a, 12b	Contaminant
Iran	<i>Ipomoea batatas</i> (L.) Lam.	Casual	1970–1980	12a, 12b	Release
	<i>Ipomoea cairica</i> (L.) Sweet	Naturalized	2002	12b	Release
	<i>Ipomoea carnea</i> s.l.	Naturalized	2002	12b	Escape
	<i>Ipomoea cocinea</i> L.	Casual	2020	12b	Release
	<i>Ipomoea hederacea</i> Anon.	Invasive	2010	12a, 12b	Contaminant
	<i>Ipomoea indica</i> (Burm.) Merr.	Invasive	2019	2, 12b	Escape/ Release
	<i>Ipomoea lacunosa</i> L.	Naturalized	2018	12a, 12b	Contaminant
	<i>Ipomoea leucantha</i> Jacq.	Naturalized	2010	2, 12a, 12b	Contaminant
	<i>Ipomoea purpurea</i> (L.) Roth	Naturalized	1949	12a, 12b	Escape
	<i>Ipomoea tricolor</i> Cav.	Casual	2010	12b	Release
	<i>Ipomoea triloba</i> L.	Naturalized	2008	12b	Contaminant

*Habitat_type_description (SynHab: www.synhab.com): 1 Forests, 2 Open forests, 3 Scrub, 4 Grasslands (4a Natural grassland, 4b Human maintained grasslands), 5 Sandy, 6 Rocky, 7 Dryland, 8 Saline, 9 Riparian, 10 Wetland, 11 Aquatic, 12 Human-made (12a Ruderal_habitats, 12b Agricultural_habitats).



Figure 1. The distribution of 14 alien *Ipomoea* taxa in Greece, Türkiye and Iran.

Ipomoea taxa in Türkiye

In the last two decades, alien *Ipomoea* taxa have been introduced to Türkiye both intentionally and unintentionally (Gönen 1999; Yazlık et al. 2014; Uludağ et al. 2017; Hançerli et al. 2018; Yazlık et al. 2018; Özkil and Üremiş 2020; Onen et al. 2023; TÜİK 2023). The genus comprises 10 alien taxa; all of which are naturalized except for *I. batatas* and *I. lobata* which are casual (Fig. 1, Table 1).

Ipomoea coccinea has been observed in disturbed areas along roads, on the edges of tea plantations, in abandoned fields, and in other wastelands (Onen et al. 2021). *Ipomoea hederacea* was first recorded in Türkiye as a weed by Gönen (1999) and has recently been recognized as a problematic plant together with *I. purpurea* and *I. triloba* in many agricultural areas: in cotton, peanut, soybean and maize fields, pomegranate and citrus (orange, tangerine) orchards and eggplant production areas (Özkil and Üremiş 2020). *Ipomoea triloba* was first recorded by Yazlık et al. (2014). This species has serious environmental and socio-economic impacts in agricultural areas, coastal areas, pastures (except arable land), stream margins, and man-made habitats (Yazlık et al. 2018). In recent years, the distribution area and population size of this species have continued to increase in the Mediterranean, Aegean, and southeastern Anatolia regions of Türkiye (Yazlık et al. 2018; Özkil et al. 2019; Özkil and Üremiş 2020; Arslan and Kitiş 2021; Doğru and Kitiş 2023). *Ipomoea hederifolia* was first recorded by Hançerli et al. (2018) and detected in maize fields in Adana province in the eastern Mediterranean part of Türkiye in 2016. *Ipomoea tricolor* has long been cultivated as an ornamental plant in gardens and some landscape areas in Türkiye (Yücel 2002; Uludağ et al. 2017), and it is found in agricultural and ruderal habitats (Onen et al. 2023).

Ipomoea purpurea was first cultivated in 1918 and is often grown in gardens, and sometimes escaping into the wild. Due to its use as an ornamental plant, it has spread rapidly in many regions in Türkiye, especially along the Mediterranean and Aegean coasts. (Hançerli et al. 2018; Özkil and Üremiş 2020). An alien record of *I. nil* in Türkiye was reported by Uludağ et al. (2017), who consider the species to be naturalized there and there is evidence that its seeds are sold as an ornamental plant in the Marmara region of Türkiye. Similarly, *I. indica* and *I. lobata* are also used for ornamental purposes in the Mediterranean region (Ercan et al. 2018; Öztürk and Görhan 2021; Esener 2023, Fig. 1, Table 1). The casual alien species *I. batatas* has been cultivated as a crop in Türkiye (Çalışkan et al. 2007; Özelçam 2013). Currently, the areas where it is grown are quite limited, reaching 35 ha in eight provinces (TÜİK 2023). No case of *I. batatas* escaping into the wild and becoming a problem has been reported.

***Ipomoea* taxa in Iran**

There are 11 alien *Ipomoea* species in Iran, of which three are casual and eight naturalized. Two of the naturalized species are invasive (Fig. 1, Table 1): *Ipomoea indica* and *I. hederacea*. The former is considered invasive as an environmental weed in open forests and natural landscapes, while the latter is more common in agricultural areas, especially in cotton, soybean, and maize production areas in northern Iran (Pahlevani and Sajedi 2011; Sohrabi and Gherekhloo 2015; Sohrabi et al. 2017; Amini et al. 2020). Most *Ipomoea* species are restricted to northern and southern Iran (Fig. 1, Table 1), where winter temperatures are above zero.

Three species have recently been recorded in northern Iran, probably introduced with cotton seed (*I. lacunosa* and *I. leucantha*) or for ornamental purposes (*I. coccinea*) (field observation by SS and JG). *Ipomoea cairica*, *I. carnea*, and *I. triloba* have been recorded in southern Iran and are restricted to ruderal habitats (Pahlevani and Sajedi 2011; Sohrabi et al. 2023a, b). *Ipomoea tricolor* and *I. purpurea* are used as ornamentals in some parts of the country (Farahmand 2018), while *I. purpurea* has been detected as a weedy species in soybean production in the Golestan and Mazandaran provinces (Savari-Nejad et al. 2010; Abbasi et al. 2022). Although *I. batatas* has an older history in the country than other species, it has not been cultivated to its full potential (Koocheki et al. 2018).

Comparison of the distribution of 14 alien *Ipomoea* in our study area revealed that the widespread species differed among the three countries. *Ipomoea purpurea* and *I. indica* were more common in Greece, while *I. batatas* and *I. triloba* were more common in Türkiye. *Ipomoea purpurea* and *I. batatas* also had a wider distribution in Iran than other species (Fig. 1). A higher number of occurrences in Greece compared to Türkiye and Iran may be attributed to the combination of a more widespread distribution and more intensive sampling in this region. Further monitoring will be essential for tracing the geographical patterns of *Ipomoea* species. Understanding alien plant species' geographical distribution and dispersal ability is important for land managers and policymakers to apply optimal management strategies (Zhou et al. 2021).

Impacts of alien *Ipomoea* taxa

In Greece, the impact of alien *Ipomoea* species mainly concerns *I. hederacea* and *I. indica*. *Ipomoea hederacea* is a troublesome weed of cotton, maize, and other spring crops (Table 2). Most problems occur in western and central Greece (Gi-

annopolitis et al. 2004; Kati and Giannopolitis 2017). It climbs on plants and causes problems in cultivation practices, especially at harvest (Drolia 2004; Giannopolitis et al. 2004). It is characterized by a high phenotypic plasticity, with its main stem reaching up to 3 m in length when growing between tall crops such as maize. In cotton, it produces long lateral shoots, with the main stem reaching up to the height of the crop. The climbing nature of *I. hederacea*, its circular movement and its ability to adapt its growth pattern according to the structure and size of the crop canopy, results in a dense network of wiry shoots surrounding the crop plants, making harvesting difficult or, in the case of dense weed infestations, impossible. It is also a strong competitor that grows rapidly, and is dispersed mainly by seed, although it can also re-sprout from stem cuttings (Kati and Giannopolitis 2006). *Ipomoea hederacea* seeds can germinate after shedding without the need for post-ripening (Kati and Giannopolitis 2006). Their germination is initiated when soil moisture is adequate, usually triggered by spring rains or irrigation of summer crops. This has resulted in dense populations of *I. hederacea* along irrigation canals, where they have outcompeted local indigenous species and served as a seed source, contaminating neighboring crops.

Reports on *I. indica* are more localized, and mainly refer to the presence of this species on the island of Rodos (Rhodes) (Dodecanese, eastern Greece). *Ipomoea indica* from the Kremasti and Mandriko areas of Rodos was found to be the host of the SPLCV virus which belongs to the sweepoviruses (Fiallo-Olivé et al. 2014). This virus can potentially infect native plants of the Convolvulaceae family, such as *I. sagittata* and *I. imperati* (Strid 2024). Galanos (2015) highlighted the presence of *I. indica* as a cause of native biodiversity loss on the island of Rodos.

In Türkiye, the most important impacts of *Ipomoea* are related to *I. hederacea*, *I. purpurea* and *I. triloba*, which cause problems in cotton and maize fields (Table 2, Fig. 2) in the Aegean, Mediterranean and Southeast Anatolian Regions (Muslu and Tepe 2016; Yazlık et al. 2018; Özkil et al. 2019; Özkil and Üremiş 2020; Arslan and Kitiş 2021; Doğru and Kitiş 2023). The climbing habit of these taxa gives them a competitive advantage over cotton and even reduces the amount of product by preventing the opening of cotton capsules (Yazlık et al. 2018; Özkil and Üremiş 2020; Arslan and Kitiş 2021). In addition, *I. triloba* causes serious problems by blocking irrigation canals in the Mediterranean region (Yazlık et al. 2018). On the other hand, *I. purpurea* and *I. hederacea* are used as ornamental plants in many regions of Türkiye. Cultivation creates a strong propagule pressure which allows the species to escape to different habitats (Table 1).

The population size of *I. hederifolia* is rapidly increasing in the Çukurova plain, one of the largest plains in Türkiye, where polyculture agriculture is practiced in the Mediterranean province of Adana. This has caused remarkable yield losses and harvest problems in maize fields (Hançerli et al. 2018).

Among other species, *I. coccinea* has a major socio-economic impact on tea plantations (Onen et al. 2021), which is the main product of the eastern Black Sea region of Türkiye and is cultivated over large areas (TÜİK 2023). Given the strong competitive ability of *I. coccinea*, supported by its high seed production and ivy-like structure, its rapid spread into different habitats and increase in population density and distribution in this region is likely. Similarly, *I. tricolor* is another species whose potential distribution in the Black Sea, Aegean, Mediterranean and some parts of central Anatolia has been predicted to increase according to a model based on germination and other traits (Onen et al. 2023).

Table 2. The detected mechanisms of impact of *Ipomoea* species in Greece, Türkiye and Iran and affected crop/native taxa.

	Species	*EICAT Criteria impact mechanism(s)	Affected crops/ native taxa	References
Greece	<i>Ipomoea batatas</i> (L.) Lam.	DD	Unknown	www.europlusmed.org
	<i>Ipomoea hederacea</i> Jacq.	1	Cotton, corn, Lucerne	Hobhouse et al. 2004; Kati and Giannopolitis 2006
	<i>Ipomoea indica</i>	1, 4, 11	Environmental weed	Fiallo-Olivé et al. 2014; Galanos 2015
	<i>Ipomoea purpurea</i> (L.) Roth	1	Agricultural and ruderal habitats	Arianoutsou et al. 2010; Balias 2014
Türkiye	<i>Ipomoea batatas</i> (L.) Lam.	8, 12	Cultivated	Çalışkan et al. 2007; Özdeş 2013; TÜİK [Turkish Statistical Institute] 2023
	<i>Ipomoea coccinea</i> L.	1, 11, 12	Tea plantations	Ercan et al. 2018; Onen et al. 2021; Öztürk and Görhan 2021
	<i>Ipomoea hederacea</i> Jacq.	1, 11	Cotton, soybean, tobacco, peanuts, potatoes, sugar beets, sunflowers, corn, orchards (citrus fruits, apples, plums, apricots, almonds, olives, dates, pomegranates, cherries, walnuts, pears, peaches, bananas, loquats), strawberries and vineyard fields	Gönen 1999; Özkil and Üremiş 2019, 2020
	<i>Ipomoea hederifolia</i> L.	1, 11	Corn	Haçerli et al. 2018
	<i>Ipomoea indica</i> (Burm.) Merr.	1, 11, 12	Gardens	Ercan et al. 2018; Öztürk and Görhan 2021
	<i>Ipomoea lobata</i> (Cerv.) Thell.	DD	Cultivated in a botanical garden	Esener 2023
	<i>Ipomoea nil</i> (L.) Roth	DD	Gardens	Uludağ et al. 2017
	<i>Ipomoea purpurea</i> (L.) Roth	1, 11, 12	Cotton, soybean, tobacco, peanuts, potatoes, sugar beets, sunflowers, corn, orchards (citrus fruits, apples, plums, apricots, almonds, olives, dates, pomegranates, cherries, walnuts, pears, peaches, bananas, loquats), strawberries and vineyard fields	Davis et al. 1988; Aykurt 2012; Muslu and Tepe 2016; Ercan et al. 2018; Özkil and Üremiş 2020; Öztürk and Görhan 2021
	<i>Ipomoea tricolor</i> Cav.	1, 11, 9	Gardens, edge of agricultural fields	Yücel 2002; Onen et al. 2023
	<i>Ipomoea triloba</i> L.	1, 9, 11, 12	Cotton, soybean, tobacco, peanuts, potatoes, sugar beets, sunflowers, corn, orchards (citrus fruits, apples, plums, apricots, almonds, olives, dates, pomegranates, cherries, walnuts, pears, peaches, bananas, loquats), strawberries and vineyard fields	Yazlık et al. 2018; Özkil et al. 2019; Özkil and Üremiş 2020; Arslan and Kitiş 2021; Doğru and Kitiş 2023
	<i>Ipomoea batatas</i> (L.) Lam.	DD	Unknown	Koochehi et al. 2018
	<i>Ipomoea cairica</i> (L.) Sweet	DD	Unknown	Farahmand 2018
Iran	<i>Ipomoea carnea</i> s.l.	DD	Unknown	Farahmand 2018
	<i>Ipomoea coccinea</i> L.	1, 11	<i>Mercurialis</i> sp.	Fields observation 2022, 2023
	<i>Ipomoea hederacea</i> Anon.	1	Cotton, corn and soybean	Pahlevani and Sajedi 2011;
	<i>Ipomoea indica</i> (Burm.) Merr.	1, 11	<i>Gleditsia caspica</i> Desf.	Amini et al. 2020; Sohrabi et al. 2023c
	<i>Ipomoea lacunosa</i> L.	1, 11	<i>Urtica dioica</i> , <i>Ficus carica</i>	Fields observation 2022, 2023
	<i>Ipomoea leucantha</i> Jacq.	1, 11	Cotton and soybean, <i>Punica granatum</i>	Fields observation 2022, 2023
	<i>Ipomoea purpurea</i> (L.) Roth	1, 6	Cotton, corn, soybean and ornamental plants	Savari-Nejad et al. 2010; Farahmand 2018; Rashidi et al. 2020
	<i>Ipomoea tricolor</i> Cav.	6	Ornamental plants	Gholamalipour Alamdari et al. 2019
	<i>Ipomoea triloba</i> L.	1	Fields and orchards	Pahlevani and Sajedi 2011

*EICAT Criteria impact mechanisms: (1) Competition, (2) Predation, (3) Hybridisation, (4) Transmission of diseases to native species, (5) Parasitism, (6) Poisoning/ toxicity, (7) Bio-fouling, (8) Grazing/herbivory/browsing, (9) Chemical impact on ecosystem, (10) Physical impact on ecosystem, (11) Structural impact on ecosystem, (12) Indirect impacts through interactions with other species (Kumschick et al. 2024). DD = data deficient.

Recently, the first analysis of the economic costs of alien species in Türkiye from 1960 to 2022 reported a total of 4.1 billion USD, the largest share of which (2.85 billion USD) was due to the agricultural sector (Tarkan et al. 2024). Considering this situation, early identification of problems caused by alien species and rapid establishment of appropriate management measures, including precautionary measures against the relevant species, would prevent future high invasion impacts (Pyšek et al. 2020a; García-Díaz et al. 2022). Therefore, the serious impacts of many of the *Ipomoea* taxa reported here in agricultural habitats (Table 1) should be taken into consideration.

In Iran, *I. hederacea*, *I. indica*, *I. purpurea*, and *I. leucantha* are the most important alien species of the genus in terms of negative impacts. *Ipomoea hederacea* and *I. purpurea* are important weeds causing yield losses in summer crops such as soybean and cotton (Pahlevani and Sajedi 2011; Abbasi et al. 2022). *Ipomoea hederacea* has been included in a list of plant quarantine pests in Iran (Anonymous 2015),



Figure 2. *Ipomoea triloba* L. in maize and citrus cultivation in Türkiye. ©Yasin Emre Kitiş.

and our survey revealed its new distribution in cotton production areas in Ardebil province. *Ipomoea indica* threatens native plants such as *Gleditsia caspica* and native grasses in the Mazandaran province (Amini et al. 2020). The field observations showed that the presence of *I. leucantha* and *I. lacunosa* reduced the growth of native plants such as *Urtica dioica*, *Mercurialis* sp., *Ficus carica* and *Punica granatum* (Table 2, Fig. 3). We observed *I. leucantha* as a new serious weed in cotton producing areas in the Kordkuy township (Fig. 4). *Ipomoea hederacea* is a troublesome weed in summer crops in Golestan (Pahlevani and Sajedi 2011; Siahmarguee et al. 2022).

The potential negative impact of the casual species of the genus in Iran (*I. tricolor*, *I. coccinea*, and *I. batatas*) needs further investigation. The allelopathic effect of *I. tricolor* is due to secondary compounds such as phenols and anthocyanins, which have been suggested as main inhibitory compounds (Gholamalipour Alamdari et al. 2019).

Impacts of *Ipomoea* species elsewhere

Many reports have highlighted the detrimental impacts of *Ipomoea* species, such as *I. purpurea*, referring to competition, allelopathy, and serving as alternative hosts for plant viruses (Crowley and Buchanan 1978; Zhang et al. 2014; Cabral et al. 2023). Negative impacts have been reported for *I. cairica* and *I. carnea*, which are globally distributed (Damir et al. 1987; Milne and Walter 2000; Liao et al. 2006; Zhao and Peng 2008; Ma et al. 2009; Takao et al. 2011; Zhu and Wu 2012; Sadek 2014). The invasion success of *I. carnea* in Egypt has been attributed to allelopathy coupled with competition for soil nutrients (Sadek 2014). *Ipomoea cairica* is recognized as the second worst invasive weed in southern China due to its extremely rapid growth, sprawling habit, and perennial life history (Wu and Hu 2004), and is predicted to become even more invasive with global warming (Wang et al. 2011). *Ipomoea batatas* is also allelopathic, but more information is needed on its potential toxicity to fauna and flora in invaded areas (Zhang et al. 2014). Hybridization, competition, and



Figure 3. *Ipomoea leucantha* Jacq., *I. lacunosa* L. and *I. indica* (Burm.) Merr. in different locations from left to right, respectively, in Golestan (1 and 2), and Mazandaran (3) Iran. ©Javid Gherekhloo.



Figure 4. The growth of *Ipomoea leucantha* Jacq. and *I. hederacea* Anon. on cotton and soybean plant to reach light and shading out crop in the north of Iran. ©Javid Gherekhloo.

disease hosting have also been reported for this species (Yang et al. 2017; Shen et al. 2019; Ouattara et al. 2023; Rifkin et al. 2023). In general, the competitive potential of alien *Ipomoea* species has been attributed to their fast growth and regeneration capacity (Sparkes and Panetta 1997; Norsworthy and Oliver 2002; Pagnoncelli et al. 2017; Averill et al. 2022; Randall 2012). *Ipomoea triloba* is a troublesome weed in many crops in the Philippines, Sri Lanka, Bangladesh, India, and Indonesia (Moody 1989; Holm et al. 1997). Reported crop losses due to different *Ipomoea* species vary from 25–90%, depending on their population density (Howe and Oliver 1987; Defelice 2001; Rizzardi et al. 2004; Price and Wilcut 2007; Bhattacharjee et al. 2009; Averilla et al. 2022; Pazzini et al. 2022). The *Ipomoea* species are ranked among the most troublesome weeds in cotton and soybean in the USA and Japan (Webster and MacDonald 2001; Norsworthy and Oliver 2002; Yasuda and Sumiyoshi 2010).

Management options

Non-chemical methods

The methods presented here rely on tools other than herbicides to reduce weed density and propagule pressure and hence the likelihood of new introductions, establishment, and spread. They include preventive, cultural, and physical (manual or mechanical) measures. Preventive measures to avoid field infestation include the use of certified crop seed and cleaning farm machinery after working in an infested field,

especially during harvest (Yazlık et al. 2019; CABI 2024). However, the efficacy of each method depends on the species (perennial species are more persistent), invasion status, habitat characteristics, and its effectiveness (Verbrugge et al. 2019; Sohrabi et al. 2023c). Indeed, the seed capsules of *I. hederacea* plants with their wiry shoots tend to cling to the combs of harvesting machines, which can then infest other fields. *Ipomoea hederacea* seeds germinate throughout the summer, leading to new infestations in the same season, and reducing the effectiveness of mechanical control methods. Although these methods are effective against annual species such as *I. hederacea*, they have no residual control effect. In addition, this species can have more than one generation in a year. For example, plants that emerge early in the season can produce seeds that can germinate soon after shedding due to the lack of dormancy and post-ripening requirements (Kati and Giannopolitis 2006). These late germinating weeds are unlikely to cause yield reduction, but they will increase the soil seed bank if left unmanaged, thus perpetuating the infestation problem. Mowing did not minimize the competition from *I. triloba* at the V4 stage (fourth leaf collar is visible) of maize, while the weeding was more effective (Giraldeli et al. 2019).

A cultivation practice to reduce weed infestation during the growing season is the stale seedbed method, which is applied prior to sowing (Shaw 1996). It relies on early tillage and favourable weather conditions to encourage the germination of non-dormant weed seeds in the top layer of soil (a few centimeters below the surface). The emerging weeds are then killed by a shallow till (or by applying a broad-spectrum herbicide such as glyphosate, i.e. combining chemical and non-chemical control methods). A small but growing number of farmers in Greece are adopting the stale seedbed approach. However, unpredictable weather conditions in recent years have reduced the chances of success for this method. Early sowing of the crop will give it a competitive advantage over *I. hederacea*, which requires a relatively warm spring temperature (above 20 °C) and adequate soil moisture for germination.

The proposed management option for alien *Ipomoea* in natural areas, such as open forests (*I. indica* and *I. leucantha* in Iran, *I. indica* in Greece), would be a combination of several mechanical control methods supporting the regeneration of native plants. Early detection and long-term monitoring have also been emphasized for effective management (Pyšek and Richardson 2010; Simberloff 2011). Jones et al. (2021) suggested biological, cultural, and mechanical tactics to effectively control *Ipomoea* plants inhabiting disturbed areas.

In recent years, flaming control trials have been carried out as an alternative and innovative approach to chemical and mechanical control against *I. triloba* in cotton, corn and banana growing areas in Antalya province. The flaming successfully controlled *I. triloba* although it depended on the application time and number of repetitions (Arslan 2022; Doğru 2023; Saruhan 2024). The method was more successful on young plants and annual species, such as *I. triloba* (Türsun et al. 2017; Arslan 2022; Saruhan 2024). Therefore, flaming application as a physical control method could potentially be used in habitats requiring control of annual *Ipomoea* species in the future.

Allelopathic plant extracts have also been used for the control of *Ipomoea*. In a study investigating the effects of ginger and turmeric extracts on the germination of *I. triloba* seeds, ginger extract reduced the germination by 97.3% and turmeric by 90.7% (Ece and Kitiş 2023). Some fungal diseases have shown promising potential for biological control of *Ipomoea* species (Soares and Barreto 2008; Nechet and Halfeld-Vieira 2019). In addition, biological control with some herbivores may also

be promising. For example, leaf-feeding herbivores can potentially be used for biological control of *I. cairica* if they consume at least 50% of the leaf biomass (Li et al. 2012). Remote sensing can be used to map and monitor these species and thereby assist in their management. Early detection and monitoring will be facilitated by using a combination of satellites and unmanned aerial vehicles (Thürkow et al. 2024).

Chemical methods

Different broadleaf herbicides were used to minimize the negative impact of *Ipomoea* species on crop production. Imazapic and the combination of mesotrione with the mixture of diuron + hexazinone resulted in better control of *I. hederifolia*, *I. nil*, *I. quamoclit*, and *I. triloba* in dry and semi-wet seasons in Brazil (Correia 2016). To control *Ipomoea* species in sugarcane, the combination of PRE and POST herbicides (e.g., 2,4-D amine or sodium salt) has been recommended (Singh et al. 2012). Herbicide control is a common method to manage *I. hederacea*, *I. lacunosa*, *I. purpurea*, *I. hederifolia*, and *I. nil* in arable crops (Toledo et al. 2017; Jones et al. 2021). Control in soybean cultivation in Japan has been difficult due to intertillage and earthing up; soil-applied herbicides are less effective than foliar herbicides (Sumiyoshi and Yasuda 2011). Bentazon, fluthiacet-methyl as foliar herbicides in soybean have shown some efficacy against *I. coccinea*, but they are less effective in controlling *I. hederacea* (Shibuya et al. 2006; Sumiyoshi and Yasuda 2011). Imazamox ammonium salt, a newly registered herbicide, is effective in controlling *I. hederacea*, despite minor damage to soybean (Asami et al. 2021). Control of *I. cairica* efficacy in China was 98% at 4.06 g/l of ethephon (Sun et al. 2015).

In Greece, the control of *I. hederacea* with herbicides, which is still the most widely applied method of weed control in arable crops in the country, has proved to be rather difficult. Field experiments in maize with the post-emergence herbicides mesotrione, bromoxynil, nicosulfuron, rimsulfuron, and the mixture rimsulfuron+thifensulfuron applied at the recommended rate gave an initial control of *I. hederacea* of over 80%. However, this efficacy was later reduced due to resprouting or the emergence of new plants (Giannopolitis et al. 2004). Further pot experiments with the above treatments and the pre-emergence herbicides prometryn, fluometuron and alachlor showed good efficacy (>80%) four weeks after application, although the efficacy of the post-emergence herbicides was transient again due to resprouting. Similarly, in another set of field and greenhouse experiments, prometryn controlled 86% of the *I. hederacea* plants, while mesotrione reduced the fresh and dry weight of the weed by more than 80% (Drolia 2004). In this study, fluometuron and dimethenamid gave poor weed control, reducing the fresh or dry weight of *I. hederacea* by 50% and 30%, for each herbicide, respectively, while bentazon and oxyfluorfen provided moderate control (58–66%). It should be noted here that the pre-emergence herbicides prometryn and alachlor, and the post-emergence herbicide bromoxynil, are no longer registered for use in Greece. The post-emergence herbicide dicamba can provide effective control of *I. hederacea* in maize as has been demonstrated in pot experiments, although under field conditions, the weed's prolonged period of emergence during the cropping season re-established the problem. It is evident from the above that a combination of methods is required for effective and long-lasting control of *I. hederacea*. This includes cultural and mechanical measures supplemented by residual pre-emergence, and effective post-emergence herbicides (Giannopolitis et al. 2004).

In Türkiye, the lack of management programmes and registered herbicides for *I. purpurea*, *I. hederacea* and *I. triloba*, which are commonly found in cotton and maize fields (Muslu and Tepe 2016; Yazlık et al. 2018; Özkil and Üremiş 2020; Arslan and Kitiş 2021), has increased their abundances and impacts. For example, *I. triloba* is the second most important weed in terms of density (29 plants/m²) and incidence (67%) in cotton production areas in the Antalya province (Arslan and Kitiş 2021). However, in recent years, the efficacy of some active ingredients against *I. triloba* has been investigated, and trifloxysulfuron sodium was found to be quite effective in cotton fields (Özkil et al. 2019; Arslan 2022).

In Iran, control is not direct but targeted with other broadleaf weeds. The infected fields are controlled by common broadleaf herbicides such as bentazon (thiadiazine group), trifluralin, ethalfluralin and pendimethalin (dinitroaniline group), envoke (trifloxysulfuron sodium) (Barati Mahmoodi et al. 2011; Gholamalipour Alamdari et al. 2016; Fakhari et al. 2020). The combination of tillage and herbicide application has recently been recommended to improve weed control in soybean and cotton fields (Gholamalipour Alamdari et al. 2016; Ghavi and Armin 2021). The correct time to control *I. purpurea* in croplands in Iran is suggested to be at the third or fourth leaf stage or at the latest before seed formation to limit its seed production and prevent regeneration of the seed bank (Abbasi et al. 2022).

In general, effective management plans require a multidisciplinary approach and stakeholder involvement (Woodford et al. 2016; Erazo et al. 2024). Prioritizing species, applying optimal long-term strategy, and sufficient economic resources to support the implementation are key elements of sustainable approaches (Epanchin-Niell 2017; García-Díaz et al. 2022; Kumschick et al. 2024).

Conclusions

All *Ipomoea* species generally have high environmental and socio-economic impacts, especially as agricultural weeds. The competitive ability of alien *Ipomoea* is expected to increase with climate change. Furthermore, the use of these species as ornamentals increases the risk of their rapid invasion and establishment in different habitats. Therefore, priority should be given to precautionary measures that minimize the risk of their invasion, such as including these species in quarantine plant lists or using certified crop seeds. In addition, farmers and the general public need to be made aware of the environmental and socio-economic impacts of *Ipomoea* species. As highlighted in our paper, the management of weedy *Ipomoea* taxa is particularly challenging in all three countries studied here and beyond. The lack of registered herbicides to control these species hinders the efforts to minimize the severity of their impacts. It is therefore imperative that non-chemical management options are developed, optimized, and combined with preventive measures, to reduce the risk of invasion and mitigate the negative impacts of alien *Ipomoea* species. Moreover, due to their ability to climb and reach heights of up to 2.0 m or more, along with its ivy structure and showy flowers, these species are popular ornamental plants. However, they can have serious impacts on many habitats, especially in agriculture. As a result, *Ipomoea* species can be considered relatively easy to be monitored. Therefore, future monitoring and distribution mapping, as evidenced in our study, is likely to be feasible, potentially involving citizen scientists. In addition, it is recommended to consider developing appropriate tools, machinery and equipment for innovative management approaches such as flame control

and electromagnetic rays currently being implemented in trials in Türkiye. Finally, since this study reflects the distribution and impact status of the relevant taxa to a large extent, it also provides an idea for issues such as prioritizing the species to be included in the risk analysis.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Data availability

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

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Supplementary material 1

***Ipomoea* taxa in Greece, Türkiye and Iran, their common names, main characteristics as alien plants and native ranges**

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Research Article

Border biosecurity interceptions for air passengers – assessing intervention methods and analytic tools

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Abstract

At-border interventions are a critical step along the biosecurity continuum, to measure and control the risks associated with the cross-border movement of people and goods. Air passengers are a high-volume pathway for a range of biosecurity risk materials, against which various interventions may be used (e.g., manual searches, detector dogs, x-rays, etc.). Tasmania is an island state, and its environment and industries have benefited from a low level of invasive pests due to their geographic isolation. Therefore, relatively strict at-border interventions are used to prevent the entry of new pests, including some serious invasive pests already present on mainland Australia (e.g., Queensland (*Bactrocera tryoni*) and Mediterranean (*Ceratitis capitata*) fruit fly). Using a large interception database for domestic air passengers entering the southern Australian state of Tasmania from mainland Australia, this study applies common statistical modelling tools to assess the efficacy of interventions (namely, dog detectors, and bag searches), and to identify pathway risk factors (e.g., flight origin/route). This analysis considered the effects of interventions on both voluntary declarations by passengers and also detections of undeclared risk material on passengers. The analysis also focused on biosecurity risk items generally (e.g., fruits and vegetables, meat products, cut flowers), and items that are specifically considered to be fruit-fly hosts. The results highlight that active at-border interventions and the presence of biosecurity inspectors capture a significant volume of biosecurity risk items at the border, and detector dogs have particularly strong positive effects on the rate of interceptions, particularly for items detected on passengers. Conducting bag searches also appears to increase interceptions, both by increasing the rate of items being detected and by encouraging voluntary declarations. Sensitivity analyses then test the robustness of results to modelling implementation methods and distributional assumptions. This study demonstrates how statistical modelling can provide robust insights into biosecurity interventions and risk factors along pathways, and further highlights the value of high-quality interception data resources for informing and improving biosecurity systems.

Key words: *Bactrocera tryoni*, border biosecurity, *Ceratitis capitata*, detector dogs, fruit fly, invasive species, passenger screening, pathway risk analysis



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Introduction

Biosecurity border interventions seek to balance the need for the cross-border movement of goods and people against their biosecurity risks. Although intervention policies inherit from a common framework of international agreements (Out-hwaite 2010), there are substantial differences in the policies and implementation

of biosecurity interventions across jurisdictions. These differences produce variation in the risk of pest introductions across states, countries and regions (Whattam et al. 2014; Epanchin-Niell et al. 2021). Australia's biosecurity system tends to have a higher appropriate level of protection and to implement comparatively stricter interventions than many other jurisdictions (Whattam et al. 2014; Black and Bartlett 2020). This is partially due to the opportunity presented by Australia's historical isolation, which has made its primary industries relatively pest-free on a global scale. However, activities such as tourism and trade have reduced and will continue to reduce this isolation, increasing the risk of pest introductions (Turner et al. 2021; Whattam et al. 2024). Recent studies estimate an aggregated cost of invasive species to Australia to be AU\$389.59 billion since the 1960s (Bradshaw et al. 2021), and a net present value of AU\$314 billion for the national biosecurity system in terms of the assets that it protects (Dodd et al. 2020; Stoeckl et al. 2023). This highlights the critical importance of ensuring that border interventions act as effective barriers against biosecurity threats.

This is particularly true for the Australian island state of Tasmania (Fig. 1). Due primarily to their geographic and evolutionary isolation, island ecosystems possess a disproportionate level of the earth's endemic species and support a large percentage of its biodiversity (Kier et al. 2009; Weigelt and Kreft 2013). Tasmania has specifically been identified as a major centre of endemism for Australian flora; for example, more than half of the 30 native *Eucalyptus* species in Tasmania are endemic (Crisp et al. 2001; Potts et al. 2016). Local industries, communities, and natural ecosystems benefit from the state's relative isolation and low levels of pests, including species that are present elsewhere in Australia, such as Queensland fruit fly ('Qfly', *Bactrocera tryoni*; Florec et al. 2013), Mediterranean fruit fly ('Medfly', *Ceratitis capitata*; Cook and Fraser 2015), tomato potato psyllid (*Bactericera cockerelli*; Moir et al. 2022), and grape phylloxera (*Daktulshaira vitifoliae*; Skinner 2018). These are potential threats for Tasmania, and a 2018 incursion of Qfly in the state's north cost millions in direct eradication costs in addition to further indirect costs (e.g., via temporary market access losses; Blake 2019). Tasmania's low-pest status is therefore a biosecurity challenge, and islands can be particularly vulnerable to impacts from invasive pests and diseases (Keitt et al. 2011; Fraser 2016; Brettell et al. 2021). Furthermore, globalisation is expected to increase movement across borders in both goods and people and to continue to bridge the geographic barriers that once kept Australia and Tasmania isolated (Dodd et al. 2015; Seebens et al. 2017, 2021).

Air passengers are an important high-volume pathway for pest introductions, with around 20 million passengers arriving annually in Australia in 2023–24 (BITRE 2024). Pests may be introduced via luggage (e.g., via infested fruit) or by being attached to passenger clothing or belongings (e.g., soil on shoes or sports equipment; McNeill et al. 2011; Pace et al. 2022; Robinson and McNeill 2022). Air passenger pathways may be particularly important for pest insects, including Medfly (Liebhold et al. 2006; McCullough et al. 2006). Passengers are also a pathway for animal diseases, for example, measures targeting African swine fever detected a yearly average of 33,684 pork products from 2% of screened passengers entering Australia from 2021/22–2023/24 (DAFF 2024). Interventions on these pathways must therefore be able to mitigate a high volume and a diverse range of biosecurity threats.

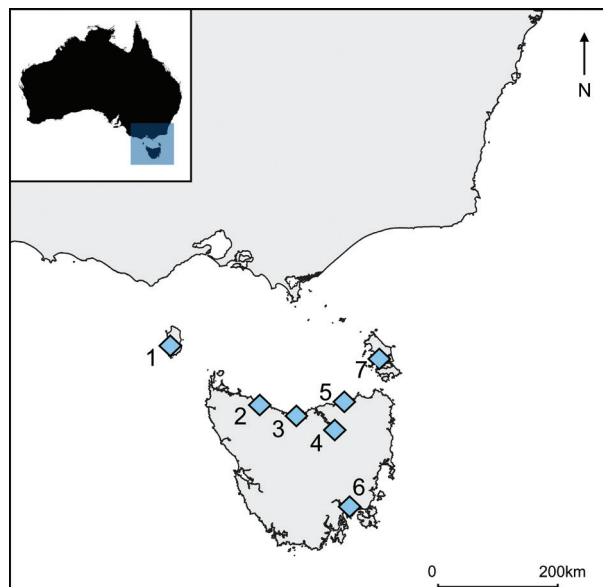


Figure 1. Location of Tasmania in relation to mainland Australia (inset), including the locations of the seven arrival ports for air passengers, namely (from left to right) 1. King Island, 2. Burnie, 3. Devonport, 4. Launceston, 5. Bridport, 6. Hobart, and 7. Flinders Island. Base map produced via QGIS (v3.24.2; www.qgis.org). (Note, airports have been anonymised for the remainder of the analysis and labelled Airport_A, Airport_B. etc.)

A range of risk mitigation tools can be employed at multiple points on the air passenger pathway, from pre-departure, in-transit and on-arrival screening/inspection phases of the biosecurity continuum (Sequeira and Griffin 2014; Whattam et al. 2014). For international arrivals into Australia, travellers are subject to pre-arrival risk profiling, and a subset of arrivals are subject to active interventions/screening at the border (Inspector-General of Biosecurity 2019). Common at-border interventions include manual examination, dog detector teams, and x-rays (Inspector-General of Biosecurity 2022). Tasmania also applies similar active interventions for domestic arrivals, including manual bag searches and detector dogs.

Detector dogs may be particularly valuable in air passenger screening, being able to screen large volumes of passengers and luggage efficiently, and able to be trained to target general biosecurity materials as well as specific pests and diseases (Whattam et al. 2014; Moser et al. 2020). Nonetheless, there are limited studies assessing their efficacy relative to other intervention methods. Furthermore, biosecurity interventions are often targeted towards specific flights to maximise the utility of limited resources, e.g., flight-based-traveller profiles used to target international arrivals into Australia (Inspector-General of Biosecurity 2019). Therefore, further empirical evidence about the relative efficacy of different interventions may help target intervention resources towards the highest-risk arrivals.

The analysis of biosecurity interception/surveillance data is prone to some common issues in statistical modelling, including zero-inflation (i.e., where data includes a large proportion of zeros, for example where detections of targeted items are rare), overdispersion (e.g., where variance is much higher than predicted), and censoring (e.g., if data is only recorded where contamination is detected; Kachigunda 2020; Turner et al. 2020; Trouvé and Robinson 2021; Kachigunda et al. 2022). Failing to account for overdispersion or zero-inflation in data can lead to

biased or inaccurate parameter or error estimates (Harrison 2014; Campbell 2021; Feng 2021). Although some studies suggest that the outputs of mixed-effects models can be robust to violations of distributional assumptions (e.g., Schielzeth et al. 2020; Knief and Forstmeier 2021), exploring the potential effects of model design and implementation may be important considerations when using interception data to inform biosecurity decision-making.

Focusing on domestic flight arrivals into Tasmania from mainland Australia, the goal of this study is to assess the efficacy of passenger interventions and pathway risk factors on biosecurity interceptions. This focuses on both general biosecurity risk material ('BRM') interceptions, and interceptions of BRM material specifically relevant to Qfly and Medfly (collectively referred to as fruit fly, 'FF'). Preventing FF incursions has been a focus of Tasmania's border biosecurity system, particularly following the 2018 incursion. This study uses recent air pathway intervention data for Tasmania. These data are rich resources for our study, as they include records for all commercial arrivals, as well as relevant data on the types/amount of BRM intercepted. This provides a valuable opportunity to apply statistical modelling approaches and assess their sensitivity to implementation methods. The specific aims of this analysis were:

1. To determine the relative effects of different interventions (namely, dog detector teams and luggage searches) on the rates of BRM and FF host interceptions, including voluntary declarations by passengers and involuntary detections of items by biosecurity officers. We did not make any specific directional predictions about the effects of searches and detector dogs on interceptions.
2. To identify pathway-risk heterogeneity based on the origin and specific routes of flights. We expected substantial variation in interception rates related to flight origin and route, which may be used to identify high-risk arrivals.
3. To test whether our results are sensitive to overdispersion and zero-inflation by implementing Bayesian mixed models with zero-inflated Poisson and negative binomial distributions. We expected the outputs of models and the estimated effects of intervention methods to be robust to different implementation approaches.

Methods

Data context and overview

Tasmania is an island state (see Fig. 1), with a cool temperate climate, unique natural ecosystems characterised by high endemism (Crisp et al. 2001; Potts et al. 2016), and a large primary industry sector with an income from agriculture, forestry and fishing industries worth around AU\$3.5 billion in 2022–23, or ~9% of the Gross State Product (ABS 2023).

The main entry pathway for domestic passengers is from flights originating in six Australian mainland states/territories, with a significant but smaller volume of maritime arrivals (e.g., ferries, cruise vessels, private vessels, etc.). Interceptions from air passengers are recorded in the Biosecurity Activity Database System (referred to as 'BAS data'), from which data from 1 January 2019–1 September 2023 were available. There are BAS interception records for 59,917 domestic in-

terstate flight arrivals, carrying over 6.5 M passengers (~1.4 M/year on average), from which 66,675 BRM interceptions were made. This study focuses specifically on domestic interstate arrivals, as these are routinely recorded in BAS data, and domestic flights represent a large majority of air arrivals in the state (~99% based on 2023/24 volumes; BITRE 2024).

BRM items are generally defined to include fresh produce (i.e., fruits and vegetables), animal products including seafood, live animals, plant material (e.g., nursery stock, seeds), and soil attached to sports equipment or clothing (DNRET 2023). Biosecurity interventions for Tasmania have a particular focus on preventing incursions of FF into Tasmania, and a large subset of BRM interceptions (43,803, or approximately 2/3) are of items considered to be FF hosts. For this analysis, FF hosts include 130 taxa listed as Medfly and/or Qfly hosts in the Plant Biosecurity Manual Tasmania 2023 (Biosecurity Tasmania 2023). For details of BRM and FF host item definitions and of intercepted BRM items, see Suppl. material 1: A.

Data processing

Data for 59,917 flight arrivals was found to be within the scope of analysis, which excludes flights from international origins or within the state, and flights with no data (i.e., cancelled, diverted, missed, or cleared remotely; ~14% of all records). A further subset of 27 arrivals was excluded because of apparent data entry issues, and 25 arrivals into one airport were excluded as no commercial flights arrive at this location, and interceptions for the remaining private arrivals were extremely low, causing computational issues with model implementation. As only a small fraction of actual arrivals is excluded, and missed arrivals do not appear to be targeted/biased towards specific arrivals, we are confident that these exclusions do not reduce the operational relevance of the analyses.

Six count variables were used as response variables, namely:

1. the total number of BRM interceptions per flight (N_Total);
2. the number of BRM declarations by passengers (N_Declarations);
3. the number of undeclared BRM interceptions (N_Detections);
4. the total number of FF host interceptions per flight (N_Total_FF);
5. the number of FF host declarations by passengers (N_Declarations_FF); and,
6. the number of undeclared FF host interceptions (N_Detections_FF).

Total BRM and FF host interceptions are the sum of their corresponding declared and undeclared detection counts. Both BRM and FF variables were used to explore how interventions perform against both general biosecurity threats as well as high-priority/high-risk biosecurity materials, respectively. Detections may occur through manual searches or via detector dogs, and biosecurity staff also ask for passengers to voluntarily declare any BRM items, both of which may occur at several stages of the arrival process (e.g., as passengers enter terminals, or in luggage collection areas). Both detections and declarations were included to explore how interventions influence both voluntary and involuntary compliance behaviour in passengers (e.g., whether detector dogs primarily increase interception through direct detections, or whether their visual presence also encourages voluntary declarations).

The number of interceptions was calculated as the sum of each distinct type of BRM or FF host, separated by the passenger (e.g., if 2 passengers are intercepted each carrying 3 types of BRM, $N_{\text{Total}} = 6$). The rationale is that each commodity type may represent a distinct biosecurity threat, as may the same kind of commodity being carried by two separate passengers.

Statistical analysis A: Intervention and pathway risk effects

Generalized linear mixed effects ('glm') models with a Poisson distribution were implemented via package 'lme4' (v1.1-33, Bates et al. 2015), in the R statistical environment (v4.2.3, R Core Team 2013). This was chosen for the primary analysis, as lme4 is an accessible package that can implement models using common distributions, relative to more complex Bayesian implementation methods that may be required for more advanced model types. Therefore, this approach may be more relevant for use by non-academic users such as biosecurity managers.

Four fixed effects were included, namely: arrival airport ("Location"), intervention regime ("Regime"), number of bag searches ("BagSearchCount"), and number of passengers per flight ("PassengerCount"). "Regime" includes five combinations of one or more dog detector team ('DDT') and biosecurity inspector ('BI'), i.e. one BI, two BIs, one DDT, one DDT with one BI, and two DDTs. Both DDTs and BIs have been deployed across all airports. Airports were included as fixed effects, as they may differ both in their interception efficacy and in the underlying rates of contamination on flights arriving at each location. Count predictor variables (i.e., passenger and bag search counts) were square-root transformed and Z-scaled to reduce skewness, to improve both model performance and the interpretability of effect estimates (per Schielzeth 2010).

Models included two random effects, to assess the level of variance associated with the flight's Australian state/territory of origin ("FlightOrigin") and specific flight route ("FlightNumber"; nested within origin). Flights without a number recorded were categorised as 'Itinerant/Other', with a large majority considered to be private non-commercial arrivals, but also likely to include a small percentage of commercial flights for which their numbers were not entered into the database. For further details of model structure see Suppl. material 1: B.

Unless otherwise stated, all values in square brackets below represent 95% confidence intervals (or credibility intervals for Bayesian models below; '95CI') for the estimated effects. The statistical significance of any fixed effects is inferred from whether their 95CIs include zero. Random effects are assessed based on how much variance is explained in models, and whether 95CIs for any specific random intercept predictions include zero. Where appropriate, parameter/effect estimates below have been converted to percentage changes in the expected number of interceptions for ease of interpretability. Marginal means were extracted from models using the package 'emmeans' (v1.8.7, Lenth 2023), to estimate expected interception rates under different intervention regimes.

Statistical analysis B: Model sensitivity

Sensitivity to overdispersion and zero-inflation was tested by re-fitting a subset of models in a Bayesian framework via the package 'brms' (v2.19.0, Bürkner 2017). From the six response variables used in the main analysis, two were selected for

sensitivity analyses. These were the total BRM interceptions (N_{Total} , i.e., the most inclusive aggregation of interception data), and the number of FF host declarations ($N_{\text{Declarations_FF}}$, i.e., the most sparse response variable).

Four alternative distributions were tested for each response variable, namely Poisson (as in the main analysis but implemented in a Bayesian framework), zero-inflated Poisson, negative binomial, and zero-inflated negative binomial, respectively. These were chosen as common alternatives to account for cases with excess zeros and overdispersion in ecology and other fields where count data is common (Lindén and Mäntyniemi 2011; Campbell 2021; Pittman et al. 2022). Models used the same fixed and random effects specifications as in the glm models, with default noninformative priors to reflect our lack of prior knowledge for parameter estimates (chains = 3, iterations = 3000, warmup = 1000). The outputs for the fixed effects of intervention regimes and bag searches and the random intercepts associated with flight origins were estimated and qualitatively compared between models.

Measures of model fit were also estimated for all models, i.e., Akaike/Watanabe–Akaike information criterion ‘AIC’/‘WAIC’ as a measure of the quality of model fit for the dataset. Marginal and conditional R^2 values as measures of the proportion of variance were explained by fixed effects and both fixed and random effects respectively (via package ‘performance’, v0.10.3, Nakagawa and Schielzeth 2013; Lüdtke et al. 2021). Overdispersion and zero-inflation tests were also conducted (also via ‘performance’).

Results

Intervention and pathway risk effects

Models identified significant effects of biosecurity interventions upon interceptions of both BRM and FF host items. Estimated BRM and FF host interception rates were significantly higher when detector dogs were present. For example, the estimated total BRM interceptions (N_{Total}) per flight with one DDT was 0.88 [95CI: 0.80, 0.96], compared to 0.50 [0.46, 0.55] for one BI when using our default Poisson model. This effect appeared to primarily be driven by increases in the number of detections, with DDTs having strong positive effects on BRM and FF host item detections (Fig. 2). Full results, code, models and outputs are available via Open Science Framework (osf.io/78tv9/; doi: 10.17605/OSF.IO/78TV9), and detailed model outputs are available in the Suppl. material 1: B.

The number of bag searches conducted (performed both by DDTs and BIs) had a positive effect on the estimated total, declared, and detected BRM counts. Unsurprisingly, the square root number of bag searches per arrival was associated with a per-unit increase in total BRM interceptions of 24.0% [23.3%, 24.6%]. A similar effect was found for BRM declarations (i.e., 23.8% [22.8%, 24.9%]) and detections (i.e., 24.1% [23.3%, 24.9%]). Similar effects were observed for FF host interceptions in total (23.8% [23.0%, 24.6%], declarations (23.2% [22.1%, 24.4%]), and detections (24.3% [23.2%, 25.4%]). In both cases, the effect was similar for detections and declarations, suggesting that conducting more bag searches increases the rate of BRM being detected and encourages more declarations. As expected, increased passenger counts were also associated with increased interception rates across all response variables. Finally, there were also some differences between arrival airports in their estimated interception rates (see Suppl. material 1: B).

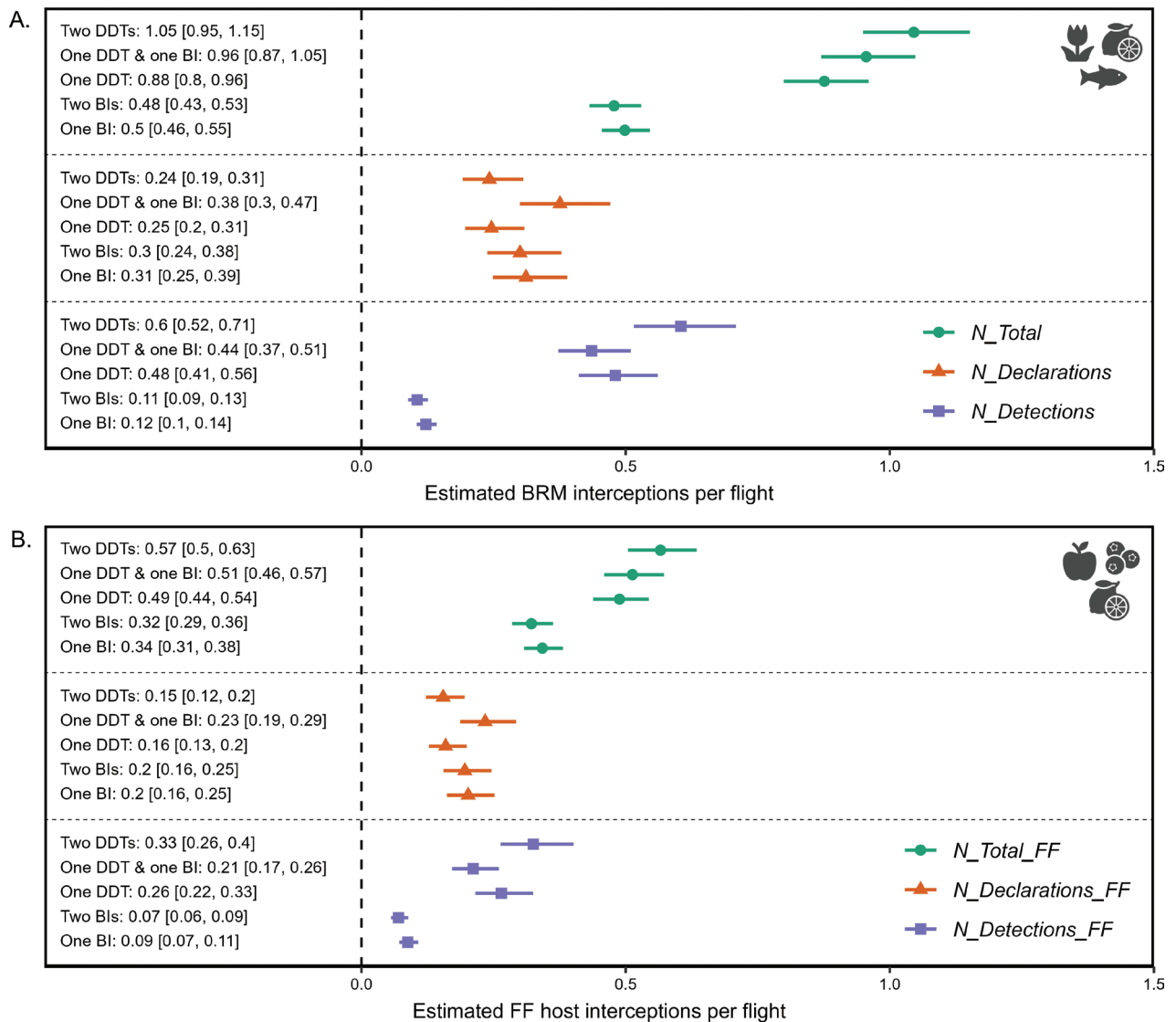


Figure 2. Estimated interception rates for air passengers under different border intervention regimes, for (A) biosecurity risk material (BRM) interceptions and (B) the subset of BRM that are fruit fly (FF) host items. Regimes include combinations of detector dog teams (DDT) and biosecurity inspectors (BI). Note, that estimated rates are the predicted number of interceptions per flight, and are independent of other factors included in the models (i.e., are estimated based on a flight with a mean number of passengers, and a mean number of bag searches, and averaged across arrival airports). Total, declared and detected rates come from distinct models, so estimates are not expected to be additive.

Random factors, flight number and flight origin, both explained some variance in interception rates (e.g., for N_Total , $V_{FlightOrigin} = 0.004$, $V_{FlightNumber} = 0.042$), although the random effects only explained a small proportion of variation relative to fixed effects (i.e., for N_Total , $R^2_{marginal} = 0.543$; $R^2_{conditional} = 0.570$, proportional $V_{FlightOrigin} = 0.002$, and proportional $V_{FlightNumber} = 0.025$). It should also be noted that the overdispersion may lead to overestimates of R^2 values (e.g., Harrison 2014), so these values should be interpreted cautiously. Nonetheless, random intercept predictions for flight origin show how pathway factors may be used to identify and target interceptions towards higher risk arrivals (Fig. 3). Similar pathway heterogeneity can also be identified for flight number (see Suppl. material 1: figs B.1, B.2).

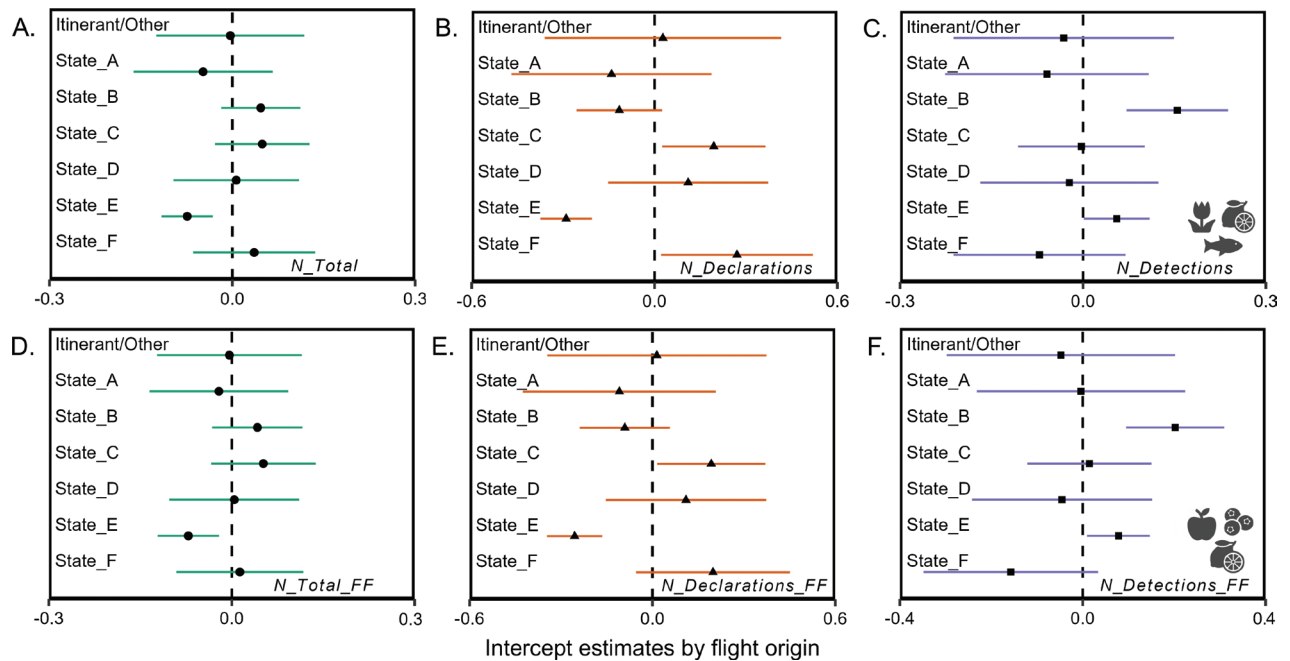


Figure 3. Predicted random intercepts by flight origin for (A–C) biosecurity risk material (BRM) interceptions, declarations, and detections; and (D–F) fruit fly (FF) host item interceptions, declarations, and detections. Error bars represent 95CIs, and intervals that do not include zero are considered to have significantly higher or lower levels of BRM or FF host interceptions than an average flight. Intercept estimates are in the modelled unit, i.e., the log of the proportional difference between the group and the overall expected BRM or FF host count.

Model sensitivity

Tests showed that models used in the main analysis for N_Total and $N_Declarations_FF$ both had probable zero-inflation (ratio of predicted to observed zeros: 0.85 and 0.92, respectively), and overdispersion was present in both cases (N_Total : dispersion ratio = 1.634, $\chi^2 = 97786.221$, $P < 0.001$; $N_Declarations_FF$: dispersion ratio = 1.709, $\chi^2 = 102303.640$, $P < 0.001$). Notably, overdispersion can be a common consequence of zero-inflation, in which case a zero-inflated Poisson approach may be sufficient to account for both issues (see Yang et al. 2009). Measures of model fit also showed that all models accounting for zero-inflation had lower WAIC scores than those that did not, whereas negative binomial models had the lowest scores and appeared to be the best-fitting models tested (see Suppl. material 1: table B.1).

Nonetheless, sensitivity analysis showed that the outputs were relatively robust to implementation methods, with the patterns identified qualitatively similar between implementation types, but with some variation in the magnitude and uncertainty of effects. Estimated interception rates under differing regimes showed similar patterns when using a Bayesian implementation, although with slightly greater uncertainty (e.g., the estimated rate with one BI was 0.50 [0.44, 0.57] compared to 0.50 [0.46, 0.55] in the main model; see Fig. 4). Incorporating zero-inflation into Poisson models led to higher rate estimates (see also Suppl. material 1: fig. B.3). Negative binomial models, which were the best-supported models based on WAIC scores, instead produced, considerably lower estimates of BRM interceptions, while still showing qualitatively similar differences when comparing interception rates between regimes.

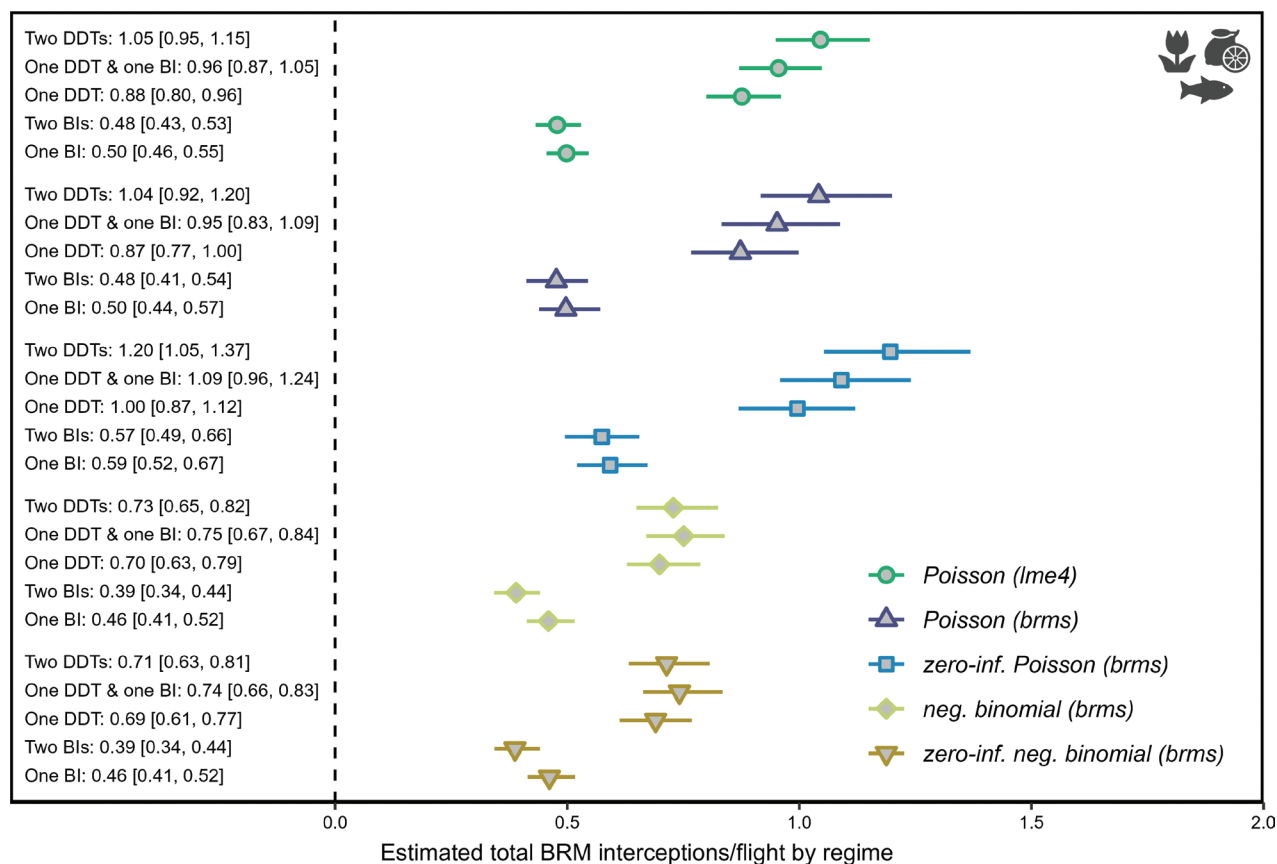


Figure 4. Estimated biosecurity risk material (BRM) interception rates for air passenger intervention regimes using different model implementations. Regimes include combinations of detector dog teams (DDT) and biosecurity inspectors (BI). Estimates are for five different model implementations, based on the modelled distribution and on a frequentist (lme4) vs Bayesian (brms) framework (see further details under Suppl. material 1: table B.1).

Random intercept predictions also appeared to show qualitatively similar patterns for FF host detections (Fig. 5) and BRM interceptions (Suppl. material 1: fig. B.4). Comparing outputs based on a Poisson distribution, the Bayesian (brms) approach produced greater uncertainty in the mean intercept estimates/predictions relative to the frequentist (lme4) implementation (Figs 4, 5), although the means themselves remained relatively consistent.

Discussion

Biosecurity interceptions at Tasmanian airports were strongly influenced by the methods used. For example, the number of bag searches conducted increased both detections and declarations from passengers. This suggests that increased effort in active at-border surveillance by officers will increase the efficacy of interventions by promoting both voluntary compliance by passengers and detections of undeclared risk items that may otherwise have been missed. Similarly, dog detector teams are increasingly deployed for border interventions along high-volume phytosanitary risk pathways, both in Australia and many other countries (Whattam et al. 2014; Inspector-General of Biosecurity 2022). Few studies have quantitatively assessed the efficacy of detector dogs, although a recent study from Williams and Sharp (2023) showed how the presence of a dog versus an officer alone can alter passenger behaviour including eye contact, gestures or interactions with the officer/dog.

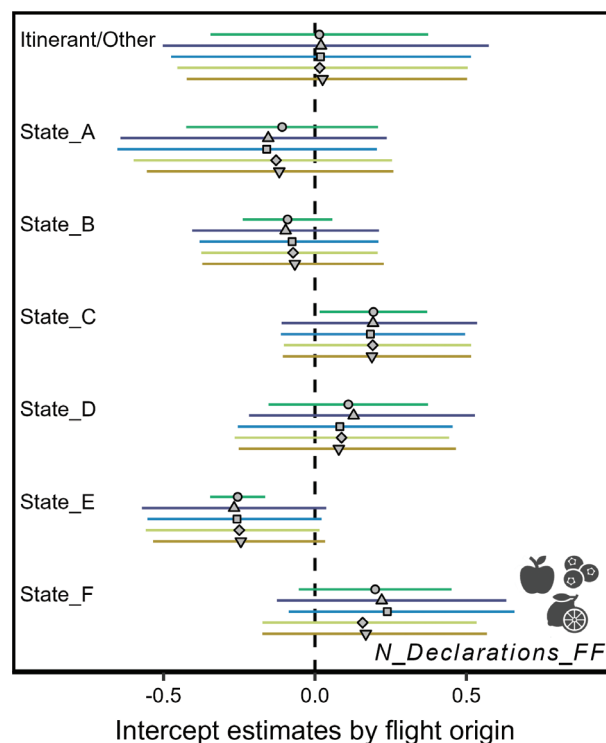


Figure 5. Predicted random intercepts for fruit fly (FF) host item detections, by flight origin. Estimates are included from five different model implementations, which from the top include the following; (green, circle) Poisson-lme4; (purple, triangle) Poisson-brms; (blue, square) zero-inflated Poisson-brms; (gold, diamond) negative binomial-brms; and, (orange, upside-down triangle) zero-inflated negative binomial-brms. Intercept estimates are in the modelled unit, i.e., the log of the proportional difference between the group and the overall expected FF host count.

Our study provides further insights into the effects dogs may have on passenger behaviour and the rate of interceptions at airports. The increase also appears to be largely driven by detections of undeclared items instead of voluntary declarations, suggesting that dogs may be particularly useful for capturing a component of the biosecurity risk material that may otherwise not be found through more passive, voluntary compliance-based methods (e.g., public awareness and education campaigns, biosecurity signage and announcements). Although notably, one inspector alone still appears to capture about half of the biosecurity risk material entering the state relative to a detector dog team. Therefore, although the efficacy of a single person is lower than the detector dog, active human surveillance still effectively mitigates a proportion of risk at the border.

The ability to analyse pathway risk heterogeneity is limited by the type of data collected for arrivals along a biosecurity pathway. Despite the relatively limited set of pathway factors included in models (i.e., origin and route) and the relatively small proportion of total variance explained by these factors, models were able to identify specific flight origins and routes as potentially either high- or low-risk arrivals. This shows how interception data may be useful for supporting risk-based approaches to interventions (e.g., Australia's flight-based-traveller profiles; Inspector-General of Biosecurity 2019) by identifying higher and lower risk arrivals to allocate limited resources to these arrivals (Trouvé et al. 2024). These analyses can provide important quantitative evidence supporting targeted resource allocations at the border, particularly when combined with further contextual information

such as pre-border pest prevalence data or estimates of potential post-border impacts. While Tasmania currently targets 100% of air arrivals, risk-based approaches could include decisions about where and when to allocate their most effective methods (e.g., detector dogs), or to potentially identify a subset of low-risk arrivals based on factors such as origin or route that can be met with less resource-demanding methods (e.g., passive interventions, signage, amnesty bins).

While these data are valuable for identifying how interventions or pathway risk influence the actual interception rates, which is critical information for implementing biosecurity interventions to reduce risk at the border, further information is required to fully quantify the risk of incursions along this pathway. For example, the risk of fruit fly establishing through the air passenger pathway would require us to estimate the actual volume of biosecurity risk material on flights and the proportion of those items infested with fruit fly (i.e., contamination/infestation rates), the proportion of risk material missed (i.e., leakage), or the viability of any individuals or larvae that may infest any of the risk material. These parameters may be estimated using complementary methods, such as endpoint surveys that target a subset of passengers as manual or X-ray searches to estimate contamination rates (Mannix et al. 2024). Samples of intercepted biosecurity risk material may also be further tested to measure their pest contamination/infestation rates. In many cases, particularly in biosecurity, empirical data is lacking. Structured expert judgement may then be used to elicit unknown parameters from relevant experts (e.g., biosecurity managers, entomologists, academics, etc.), using advanced methods to directly elicit uncertainty in parameter estimates and incorporate this uncertainty into the decision-making process (Hemming et al. 2018; Bau et al. 2024). Therefore, although this study highlights the value of interception data for informing biosecurity practices, additional knowledge is required to more completely assess and quantify risk across a biosecurity continuum.

This study used a simple and common approach for count data (i.e., regression modelling based on a Poisson distribution), performed with modelling tools that are accessible, and relatively easy to implement. Sensitivity analysis suggested that the outputs of this approach were qualitatively similar to approaches using more advanced tools (i.e., Bayesian modelling methods), or distributions (e.g., that account for zero-inflation). However, there were some notable differences in outputs. For example, interception rates estimated from negative binomial models tended to be lower, suggesting that failing to account for over-dispersion may lead to overestimates. Furthermore, these models accounting for both zero-inflation and overdispersion (i.e., zero-inflated negative binomial models) were the best-fitting models tested. Also, Bayesian methods tended to lead to higher uncertainty estimates around fixed- and random-effect parameters, so may represent a more conservative approach to modelling pathway risk factors. Therefore, while our conclusions were generally robust to implementation methods, sensitivity analysis may also be a valuable step for providing additional information for decision-makers about the robustness of any conclusions drawn from modelling and future researchers should consider their implementation. In this case, however, the operational interpretation of the simpler models was borne out by the more complex models, i.e. that detector dogs were more effective than biosecurity inspectors.

Finally, effective at-border interventions are a key step in the biosecurity continuum. As at-border interventions become more sophisticated and widely im-

plemented, large interception data sets will inevitably become more available to researchers and biosecurity decision-makers. This study highlights how this data can be a valuable resource for informing management decisions for Tasmania and can provide empirical evidence to support the implementation of risk-based approaches or the use of specific methods such as detector dogs, which can improve resource allocations and lead to more effective interventions at borders.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.


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Author contributions

NPM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Resources, Validation, Visualization, Writing – original draft. AMH: Conceptualization, Methodology, Supervision, Writing – review & editing. APR: Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing.

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Data availability

For privacy and operational purposes, all identifying information has been anonymised from datasets, including airport names, flight numbers, etc. Fully anonymised datasets, analysis code, models and outputs are all available at the Open Science Framework (osf.io/78tv9/; doi: 10.17605/OSF.IO/78TV9).

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Supplementary material 1

Additional supporting materials include the following data and modelling outputs

Authors: Nicholas. P Moran Anca M. Hanea, Andrew P. Robinson

Data type: pdf

Explanation note: (A) Composition of BRM and FF host interceptions; (B) Supplementary model design and output details.

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Research Article

Invasive *Prunus serotina* vs. *Robinia pseudoacacia*: How does temperate forest natural regeneration respond to their quantity?

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Abstract

Invasive trees negatively impact forests, by making the vegetation more homogeneous when invaders are present than when they are absent. Here, we aim to more deeply understand the effects of invasive trees on forests with a focus on seedlings and saplings and how they respond to continuous variation in aboveground biomass of invaders rather than presence/absence. Our findings are useful for close-to-nature silviculture, as they elucidate how much natural regeneration will change under particular biomasses of invasive species. Specifically, we evaluate the relationships of two invasive tree species: black cherry *Prunus serotina* Ehrh. and black locust *Robinia pseudoacacia* L. with natural tree regeneration in temperate forests. We established 160 circular 0.05 ha plots in western Poland managed forests, in two different habitat types: nutrient-poor with *Pinus sylvestris* L. and nutrient-rich with *Quercus* spp. We assessed natural regeneration by counting all trees < 1.3 m in height, within four circular subplots ($r = 3$ m). Relationships between invader biomass and regeneration of other tree species were idiosyncratic. Natural regeneration of dominant forest-forming tree species (*P. sylvestris*, *Quercus petraea*) decreased with increasing invader biomass, while shade-tolerant, nitrophilous tree and shrub regeneration increased with invader biomass. The most negatively correlated were *P. sylvestris* in nutrient-poor habitats and *Q. petraea* in both nutrient-poor and rich habitats. We observed increased density of other non-native species as *R. pseudoacacia* abundance increased, in line with the invasional meltdown hypothesis.

Key words: Advance regeneration, black cherry, black locust, invader aboveground biomass, invasion ecology, *per capita* effect, saplings, seedlings



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Introduction

Regeneration is a crucial element of forest stability and continuity (Oliver and Larson 1996; Baraloto et al. 2005; Käber et al. 2023). This process occurs through planting or sowing — artificial regeneration, or naturally without human impact — natural regeneration (Jaworski et al. 2007; Nyland 2007). Natural regeneration is essential in natural forests, without human impact, but also plays an increasing role in managed forests. Using natural regeneration of forests is part of what is called close-to-nature, ecological forestry (Batavia and Nelson 2016; Palik and D’Amato 2017). Closer to nature forestry is based to a greater extent on the natural dynamics of tree stands, which results in an increase in the complexity of their structure and increased biodiversity (European Commission 2023). Naturally regenerated stands are characterized by higher genetic diversity than forest

plantations. Such stands are therefore characterized by greater resistance and greater adaptation to local environmental conditions (Jaworski et al. 2007; European Commission 2023). Additionally, natural regeneration is more cost-effective than artificial regeneration (Oluwajuwon et al. 2024).

The natural regeneration of forests is shaped by abiotic and biotic factors. Abiotic factors include climate (Canham and Murphy 2017), light availability (Minotta and Pinzauti 1996; Modrý et al. 2004), and soil characteristics (Minotta and Pinzauti 1996; Madsen and Larsen 1997; Modrý et al. 2004). Biotic factors include diseases (e.g., Bakys et al. 2009; Lygis et al. 2014; Turczański et al. 2021), herbivory (Ammer 1996; Bruinderink and Hazebroek 1996; Iszkuło et al. 2013; Borkowski et al. 2017; Szwagrzyk et al. 2020) and competition with other plants (Mölder et al. 2019; deGroot et al. 2022; Li et al. 2023). Disturbances, including fire (Il'ichev et al. 2011) and windthrows (Szwagrzyk et al. 2018) can create gaps in the forest that alter both the abiotic and biotic environment and provide good conditions for the growth of young trees. Additionally, natural regeneration relies on seed availability (Howe and Smallwood 1982; Bartlow et al. 2018; Czortek et al. 2024). Finally, natural regeneration processes are influenced by human activities related to forest management, e.g., timber harvesting (Tavankar et al. 2017; Picchio et al. 2020), post-disturbance management (Marcolin et al. 2019), and climate change (Boucher et al. 2020; Enríquez-de-Salamanca 2022). Thus, natural regeneration depends on many factors, abiotic and biotic, natural and influenced by humans. Many of these factors that influence forest regeneration can be further shaped by invasive species, which alter both the abiotic and biotic environment.

Invasive trees and shrubs are well known for their ability to transform the recipient ecosystem (e.g., Crooks 2002; Corenblit et al. 2014; Jagodziński et al. 2024), e.g., by changes in nutrient cycling and decomposition (Aerts et al. 2017; Horodecki et al. 2019) or light availability (Starfinger et al. 2003; Dyderski and Jagodziński 2019; García et al. 2023). Those transformations can also impact the understory, including saplings and seedlings (Fuentes-Ramírez et al. 2011; Terwei et al. 2013; Dyderski and Jagodziński 2020; Langmaier and Lapin 2020). A review of studies assessing the impact of invasive plants on natural regeneration (Langmaier and Lapin 2020) identified 74 studies in Europe. Most of these studies evaluate natural regeneration based on cover and have revealed important impacts of invasive species (e.g., Maskell et al. 2006; Hejda 2012; Petrášová et al. 2013; Tinya et al. 2019). Cover, though important, does not provide reliable estimates of population size, and thus additional research on the impacts of forest invaders using precise counts of seedlings and saplings will aid in projecting forest health into the future (Terwei et al. 2013). Additionally, most studies on forest invasion compared invaded stands with uninvaded ones (Gentili et al. 2019; Lanta et al. 2022; Slabejová et al. 2023). While this is an important first step, understanding the effects of invader abundance, i.e. the *per capita* effects of invasive plants will provide more actionable information for forest management. This has only rarely been done for either herbaceous plants (Czortek et al. 2023; Wiatrowska et al. 2023) or invasive woody plants (Chabrierie et al. 2008; López-Núñez et al. 2017; García et al. 2023; Bury and Dyderski 2024b, 2024a; Jagodziński et al. 2024) with a focus on biodiversity or ecosystems services. However, to our knowledge, there are no studies assessing the effects of invader abundance on native species natural regeneration.

To address these knowledge gaps, we investigated the relationship between forest natural regeneration and the abundance of two invasive tree species, *Prunus serotina*

and *Robinia pseudoacacia*. To capture various environmental contexts, we focused on two forest types dominated by either *Pinus sylvestris* or *Quercus* spp.

Prunus serotina and *Robinia pseudoacacia* differ in their biology and ecology. Both are native to North America and were introduced to Europe in the 17th century as ornamental trees. In the following centuries, they were planted by foresters as soil-improving and wood-production trees (Starfinger et al. 2003; Cierjacks et al. 2013). Currently, *P. serotina* (Starfinger et al. 2003) and *R. pseudoacacia* (Sádlo et al. 2017; Vítková et al. 2017; Slabejová et al. 2023) are common invasive trees in Central Europe. *Prunus serotina* is mostly a shrub or small tree found in gaps (Godefroid et al. 2005; Closset-Kopp et al. 2007, 2011). It is mostly dispersed by mammals (Kurek et al. 2024), birds, or gravity (Starfinger et al. 2003; Deckers et al. 2008). *Prunus serotina* increases the soil nutrient pool, compared to native tree species, due to the higher leaf nutrient content and decomposition rate (Aerts et al. 2017; Horodecki et al. 2019). *Robinia pseudoacacia* is a pioneer tree associated with big, open patches that attains large size and occurs in the highest forest strata (Cierjacks et al. 2013; Bury and Dyderski 2024b), associated with big, open patches. Seeds of *R. pseudoacacia* are dispersed by wind and gravity (Vítková et al. 2017), though much of its spread is vegetative (Bouteiller et al. 2023). As a tree in the Fabaceae, *R. pseudoacacia* creates symbiosis with nitrifying bacteria (Rice et al. 2004; Vítková et al. 2017) and thus delivers a large amount of nitrogen to the soil largely through leaf litter (Rahmonov 2009).

We address five hypotheses in our work. (H1) We hypothesized that patterns of forest regeneration will differ in association with the two invaders. We assume that *R. pseudoacacia* and *P. serotina* will shape interactions among species and their environment in different ways, which will be manifested by different patterns of natural regeneration densities (Dyderski and Jagodziński 2020; Langmaier and Lapin 2020). (H2) We expected the regeneration of trees and shrubs to vary with invader abundance in species-specific ways (Terwei et al. 2013; Dyderski and Jagodziński 2018). (H3) Likewise, we hypothesized that there would be differences between nutrient-rich and nutrient-poor sites (Chmura 2004; Halarewicz 2011). (H4) We hypothesized that other non-native tree species may have higher regeneration densities in the presence of studied invaders, according to the invasional meltdown hypothesis (Simberloff and Holle 1999). Finally, we aimed to compare patterns obtained using three different statistical approaches (ordination, Threshold Indicator Taxa Analysis, and generalized linear mixed-effects models) to provide insights into which is best suited to the type of data we collected. (H5) We hypothesized that these three methods would provide consistent results regarding the effects of studied invaders on particular tree species' natural regeneration.

Methods

Study area and study design

We conducted the study in managed forests in western Poland, in five forest districts: Babki, Czarniejewo, Jarocin, Konstantynowo, and Łopuchówko (Fig. 2). We located study plots between 51°59'4.08"N and 52°40'9.36"N and 16°35'28.98"E and 17°37'13.26"E, in two geographical regions: the Greater Poland Lakeland (northern part) and Greater Poland Lowland (southern part). The climatic conditions are similar in the study area with an annual temperature of 8.5 °C and mean annual precipitation of 500–550 mm (BDL 2024).

We aimed to sample a quantitative gradient of invader biomass. To obtain a range, we selected study plots based on invader cover, which is straightforward to estimate, and then, after plots were chosen, we quantified aboveground biomass, following established methods (Bury and Dyderski 2024b), and described in more detail below. During initial plot selection we search for control plots (zero individuals of studied invaders ≥ 1.3 m height), medium ($< 30\%$ cover), and high ($> 50\%$) cover areas. Therefore, in our plots there could have been *P. serotina* or *R. pseudoacacia* individuals shorter than 1.3 m (included in the natural regeneration survey), however, they were rare as the density of studied neophytes depends on the proximity of propagule sources (Dyderski and Jagodziński 2018). When calculating the gradient of invader biomass, we accounted only for individuals taller than 1.3 m, and the biomass of those few individuals in the regeneration layer in control plots was negligible. We stratified our samples into two habitat types: nutrient-rich habitats that are typical of the invasive species in their native range, and nutrient-poor, where invaders had been massively introduced to improve these habitats (Starfinger et al. 2003; Cierjacks et al. 2013). Nutrient-poor sites included *Leucobryo-Pinetum* W. Mat. (1962) 1973 communities or secondary *P. sylvestris* forests. In our study, nutrient-rich sites include different subtypes of *Galio sylvatici-Carpinetum betuli* Oberd. 1957 communities or secondary *Quercus* spp. forests. Some areas had characteristics of poorer communities or slightly more fertile ones, with species characteristic of *Potentillo albae-Quercetum* Libb. 1933 or *Quercu-roboris Pinetum* Mat. et Polak. 1955 s.l. We also included two management contexts: stands in the middle of rotation age (medium age) and close to rotation age (mature age), as these age classes differ in light conditions beneath stand canopies. Stands in the middle age and those of close to rotation age differ in structure and growth dynamics (Jiang et al. 2017; Li et al. 2024). Middle-aged stands are characterized by a rapid increase in biomass, while stands close to rotation age are characterized by maximum biomass, but its increment decreases with age (Jiang et al. 2017; Li et al. 2024). Stand age is related to light availability by stem density and canopy closure, as well as in terms of higher species richness of forest specialists, related to a longer time since disturbance (Jagodziński and Oleksyn 2009; Felton et al. 2010; Conradi et al. 2020). In total, we established 160 plots (500 m² per plot), including 32 control plots (8 replications \times 2 habitat types \times 2 stand age classes), 64 plots with *R. pseudoacacia* (8 replications \times 2 invasion levels \times 2 habitat types \times 2 stand age classes) and 64 plots with *P. serotina* (same as *R. pseudoacacia*) (Fig. 1). Plots representing the same plot variant (invader \times invasion level \times habitat \times stand age) were a minimum of 5 km apart to reduce spatial autocorrelation.

Invasive species quantitative gradient – aboveground biomass

We estimated invader biomass of 102 plots in the autumn of 2021 and 2022, measuring the diameter at breast height (DBH) of all the individuals in the plots following García et al. (2023). The other 58 plots were sampled in autumn 2022 and 2023. For these, we measured the diameter at the breast height only on trees larger than 5 cm, and we counted trees thinner than 5 cm by species. Then, from the database of the 102 plots, we calculated the average DBH of individuals thinner than 5 cm by species (Suppl. material 1: table S1 for mean and SD values). This approach should not affect the validity of the



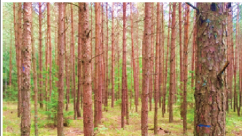
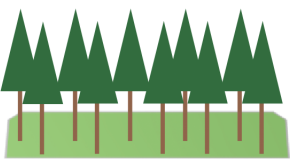



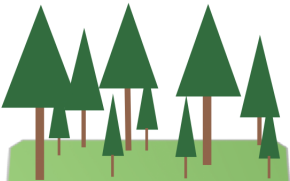


	INVADER	INVASION LEVEL	HABITAT	STAND AGE
8 REPLICATIONS	<i>P. serotina</i> 	Medium (<30%) 	 <u>Low</u> (nutrient-poor habitats with Scots pines)	 <u>Medium</u> (40-80 years old)
	<i>R. pseudoacacia</i> 	High (>50%) 		
	<u>Control</u> 	<u>Control</u> (0%) 	<u>High</u> (nutrient-rich habitats with oaks)	<u>Mature</u> (Scots pine: 80-120 years old; Oaks: 100-140 years old)

Figure 1. Scheme of the study design. Photos: S. Bury.

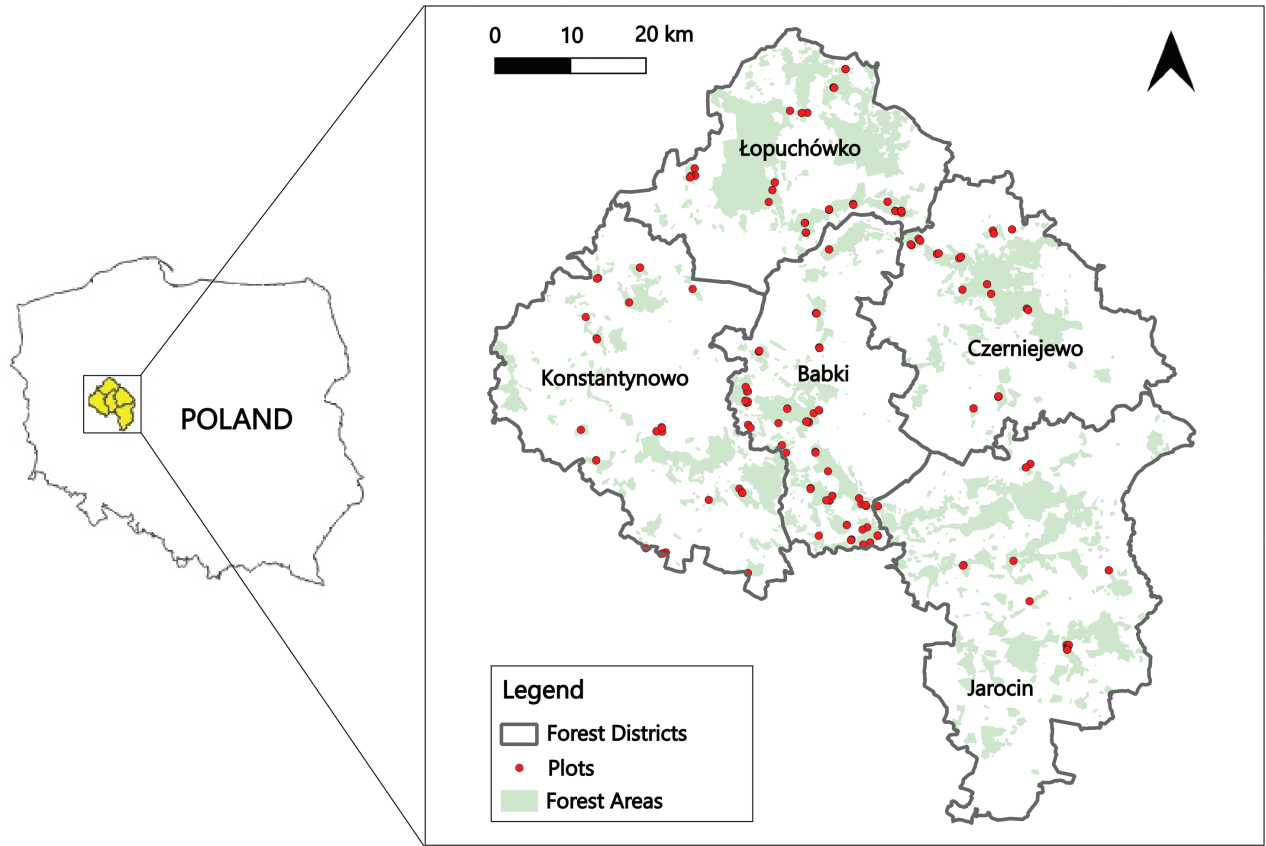


Figure 2. Distribution of the study plots (n = 160). The background map of forest cover comes from the Forest Data Bank (BDL 2024).

results, and indeed is more detailed than previous studies that either omitted trees with DBH < 5 cm or used a DBH midpoint for smaller trees (Dyderski and Jagodziński 2021a). Then, we used published allometric formulas (Suppl. material 1: tables S2–S3) to calculate the aboveground biomass for individual trees and stands (Brown 1976; Alberti et al. 2005; Forrester et al. 2017; Zasada 2017; Jagodziński et al. 2018, 2019).

Assessment of natural regeneration

In the summers of 2021, 2022, and 2023 we counted natural regeneration on four schematically distributed subplots with a 3 m radius ($4 \times 28.26 \text{ m}^2 = 113.04 \text{ m}^2$). The centers of the subplots were systematically set at 4.21 m (1/3 of the main plot radius) from the center of the plots in the four cardinal directions (N, E, S, W), using a compass and measuring tape (Fig. 3). Within these subplots we identified and counted all individuals of trees and shrubs < 1.3 m height, similarly to Kerr and Mackintosh (2012) and Mousavi et al. (2012). For each plot, we identified all seedlings germinated in the study year by species, as well as all saplings up to 1.3 m in height. Saplings may have been the product of prior years' seedlings, or clonal propagation. We treated all saplings growing separately from the soil as single individuals (Radtke et al. 2013).

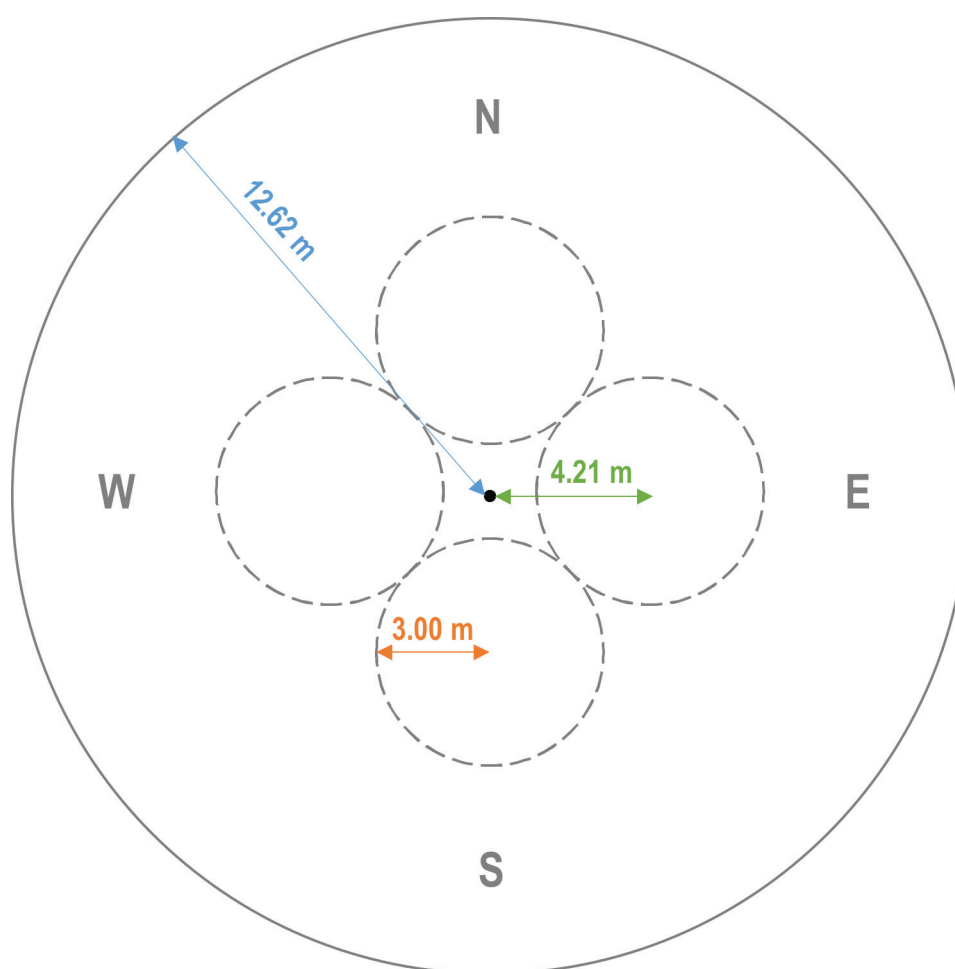


Figure 3. Schematic of the distribution of the subplots (four dashed line circles, counts of natural regeneration) within each plot (solid line circle, stand structure measurements). Plot area = 500 m^2 ($r = 12.62 \text{ m}$), subplots area = $4 \text{ subplots} \times 28.26 \text{ m}^2 = 113.04 \text{ m}^2$ ($r = 3 \text{ m}$).

Data analyses

All analyses were conducted in R (R Core Team 2023). Because one of the plots with *P. serotina* in the stand (*P. serotina* aboveground biomass = 47.11 Mg ha⁻¹; Table 1) gave a strongly biased result, we excluded it from all analyses. The aboveground biomass of *P. serotina* trees in this area was about three times higher than the next highest result. Due to differences in species pools in each habitat type, we separately analyzed the relationships with the natural regeneration for both studied invaders on both habitats. We accounted for different stand development phases, including stand age as a covariate in analyses.

We used Canonical Correspondence Analysis (CCA) to compare the effect of invader biomass and stand age. We used the `cca()` function from the `vegan` package (Oksanen et al. 2018) to develop CCA. Due to differences in sapling density among species and plots, we log-transformed data using the `decostand()` function from the `vegan` package (Oksanen et al. 2018). Furthermore, we added invader aboveground biomass and stand age as constraints. We used the `step.cca()` function from the `vegan` package to choose the optimal set of predictors based on Akaike’s Information Criterion. To assess the significance of constraints, we ran a permutation-based ANOVA-like test, implemented in the `anova.cca()` function. We visualized the results using the `ggplot2` (Wickham 2016) and `ggrepel` (Slowikowski 2024) packages.

Furthermore, we used Threshold Indicator Taxa Analysis, implemented in the TITAN2 package (Baker et al. 2023) to check which sapling species increase or decrease with invader biomass. We log-transformed biomass using $\log(x+1)$ transformation, to include also plots with zero invader biomass. For species with purity and reliability ≥ 0.95 we visualized results using the `plot_taxa_ridges()` function with default settings. We conducted the analysis using only species that occurred in at least three plots. We did not use any additional filters in the settings. The `plot_taxa_ridges()` function generates graphs on which the x-axis represents an environmental factor. In our case, this is the aboveground biomass of an invasive species ($\log(x+1)$ transformation was used). Changes in the abundance of individual taxa were assessed along the gradient of the environmental variable. In our case, this was the number of saplings of individual species on the plot. Ridges are generated for species that have achieved purity and reliability of 95%. These ridges look different for each species. The shape of the ridge tells us where in the environmental gradient a given species achieves the highest probability of occurrence.

Table 1. General characteristics of the studied plots: stand age, total aboveground biomass, invasive tree species aboveground biomass. *Quercus* — nutrient-rich habitats with *Q. petraea/robur*, *Pinus* — nutrient-poor habitats with *P. sylvestris*.

	Stand age [years]				Total Aboveground Biomass [Mg ha ⁻¹]				Invader Aboveground Biomass [Mg ha ⁻¹]			
	Min.	Mean	SD	Max.	Min.	Mean	SD	Max.	Min.	Mean	SD	Max.
Control												
<i>Quercus</i>	47	93.75	33.28	139	157.38	278.74	100.16	507.92	0.00	0.00	0.00	0.00
<i>Pinus</i>	50	76.00	22.82	117	142.95	187.17	34.50	254.63	0.00	0.00	0.00	0.00
<i>Prunus serotina</i>												
<i>Quercus</i>	44	90.31	31.96	137	138.12	267.52	93.24	505.01	0.19	6.68	7.24	27.39
<i>Pinus</i>	45	71.59	21.78	108	142.37	196.99	33.25	256.66	0.18	7.34	8.75	47.11
<i>Robinia pseudoacacia</i>												
<i>Quercus</i>	42	94.56	34.24	139	147.63	317.01	141.48	709.91	0.82	50.77	70.37	278.24
<i>Pinus</i>	42	76.81	23.06	117	125.32	182.14	31.44	246.52	0.22	20.91	31.69	153.00

The top of the ridge indicates the value of the environmental indicator for which the abundance of the species is the highest. The greater the width of the ridge, the greater the discrepancies in the data. Taxa are divided into two groups. Species whose abundance increases along the environmental gradient are marked in red (increasers), while species whose abundance decreases along the environmental gradient are marked in gray/blue (they are called decliners). The z-score value indicates the strength of the impact of a given factor or, in other words, the higher the z-score, the higher the indicator value of a given species. The higher the z-score, the darker the red or gray color (for low values it is light blue). The function also generates black vertical lines on the graph. These are the so-called threshold values, which tell us where on the gradient there is a sharp change (an increase in increasers or a decrease in decliners) in the abundance of a given species.

Finally, we used Generalized Linear Mixed-Effect Models (GLMMs), using the `glmmTMB` package (Brooks et al. 2017), with Poisson or negative binomial family distribution, to exactly determine the relationships between the abundance of seedlings and saplings of each species with invader aboveground biomass. To test the invasional meltdown hypothesis, we also evaluated the relationships between the abundance of saplings of invasive species (excluding the dominant invader whose effects we were exploring) and invader aboveground biomass. For saplings we created models for species that occurred in at least 20% of the plots in a given variant (invader and habitat type) (Suppl. material 1: table S4). For seedlings we developed models for species that occurred in at least 10% of the plots in a given variant (invader and habitat type) (Suppl. material 1: table S13). In the Results section we present only statistically significant results (with $p < 0.05$). We used the DHARMA package (Hartig 2022) to conduct formal zero inflation and dispersion tests for each model. We started from models assuming Poisson distributions, due to the count character of our data. If we did not find problems with overdispersion we tested zero inflation, and in the case of statistically significant zero inflation, we used zero-inflated Poisson distribution. If we found statistically significant overdispersion we used negative binomial distribution, adding zero-inflation when necessary. We used invader aboveground biomass and stand age as fixed continuous effects and forest district and the year of natural regeneration assessment as random intercept, to cover spatial and temporal dependence within our dataset. We used the `dredge()` function of the MuMIn package (Bartoń 2017) to choose the best model according to comparing Akaike's Information Criterion of null model $AICc_0$ with the final model $AICc$. We presented the results using marginal responses implemented in the `ggpredict()` function from the `ggeffects` package (Lüdtke 2018). These responses show mean model prediction for each level of predictor, assuming remaining predictors at a constant (mean) level, and excluding random effects (prediction for global population). We excluded two outlying observations in the model of *Cerasus avium* saplings density for *R. pseudoacacia* in rich sites (densities: 304 and 37 ind.) and one in the model of *Carpinus betulus* saplings density for *P. serotina* rich sites (density: 1206 ind.). In these plots very high regeneration density resulted from an abundance of propagule pressure in proximity and did not allow for developing models reflecting overall conditions. We used the `ggplot2` package (Wickham 2016) to present results on the graphs. In the results, we provide extreme values of sapling density for some species, i.e. zero and a value close to the maximum of the gradient for *R. pseudoacacia* and *P. serotina* in individual habitats. All mean values are followed by \pm SD, except \pm SE in the results of GLMMs.

Results

Within 160 plots, we recorded 56 woody plant species in the saplings, including 12 alien species. For seedlings, we recorded 21 woody plant species, including four alien species. We counted from 5 to 2594 saplings on particular plots with an average of 142 ± 270 individuals. We counted from 0 to 243 seedlings on particular plots with an average of 13 ± 35 individuals. The stand age on our plots varied from 42 to 139 years old for *Quercus* spp. stands and from 42 to 117 years old for *P. sylvestris* stands. The mean total aboveground biomass for nutrient-poor sites with *P. sylvestris* was very similar between control plots ($187.17 \pm 34.50 \text{ Mg ha}^{-1}$) and plots with *P. serotina* ($196.99 \pm 33.25 \text{ Mg ha}^{-1}$) and *R. pseudoacacia* ($182.14 \pm 31.44 \text{ Mg ha}^{-1}$). In the case of the *Quercus* spp. stands the average total aboveground biomass of the control stand ($278.74 \pm 100.16 \text{ Mg ha}^{-1}$) was similar to the stand with *P. serotina* ($267.52 \pm 93.24 \text{ Mg ha}^{-1}$) but stands with *R. pseudoacacia* ($317.01 \pm 141.48 \text{ Mg ha}^{-1}$) had slightly higher biomass (Table 1). The differences between *P. serotina* and *R. pseudoacacia* were visible in their biomass. For *P. serotina* we reached aboveground biomass from 0.18 to 47.11 Mg ha^{-1} with an average of $7.34 \pm 8.75 \text{ Mg ha}^{-1}$ on nutrient-poor sites with *P. sylvestris* and from 0.19 to 27.39 Mg ha^{-1} with an average of $6.68 \pm 7.24 \text{ Mg ha}^{-1}$ on nutrient-rich sites with *Quercus* spp. For *R. pseudoacacia*, we reached aboveground biomass from 0.22 to 153 Mg ha^{-1} with an average of $20.91 \pm 31.69 \text{ Mg ha}^{-1}$ on nutrient-poor sites with *P. sylvestris* and from 0.82 to $278.24 \text{ Mg ha}^{-1}$ with an average of $50.77 \pm 70.37 \text{ Mg ha}^{-1}$ on nutrient-rich sites with *Quercus* spp. (Table 1, Fig. 4). *Prunus serotina* occurred only in the understory and subcanopy layers. The largest measured individual of *P. serotina* reached a DBH of 31.1 cm and a height of 19.0 m. *Robinia pseudoacacia* occurred in the understory, subcanopy, and canopy layers. The largest *R. pseudoacacia* individuals reached a DBH of 64.2 cm and a height of 32.0 m.

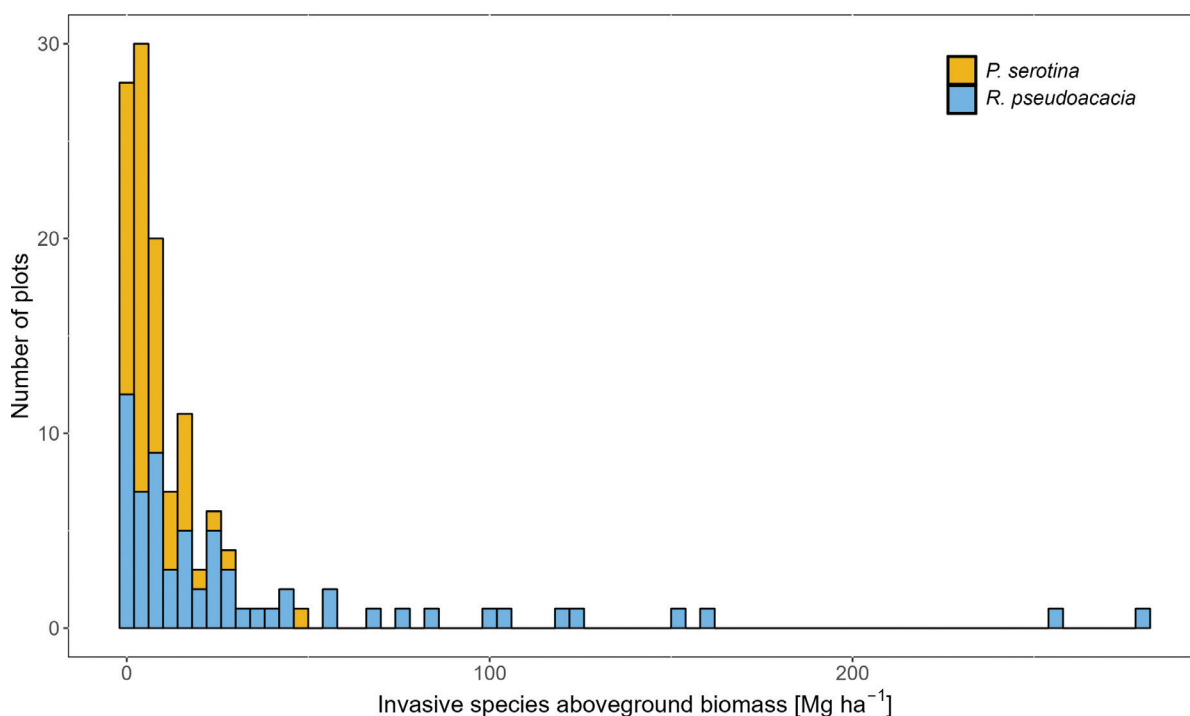


Figure 4. Histogram showing the distribution of invasive species aboveground biomass [Mg ha^{-1}] in plots with *P. serotina* ($n = 64$), and plots with *R. pseudoacacia* ($n = 64$). In this graph we excluded control plots ($n = 32$) with no studied invasive species for clarity.

Relationship between the biomass of invasive trees on species composition the natural regeneration species composition (CCA)

Species composition depended on invader biomass, both for stands with *P. serotina* and *R. pseudoacacia*, and both on the nutrient-rich and nutrient-poor sites. The stand age was statistically significant for *R. pseudoacacia* on nutrient-poor ($p = 0.004$ Fig. 5, Table 2) and nutrient-rich sites ($p = 0.034$). *Prunus serotina* biomass was positively correlated with the frequency of *Sorbus aucuparia*, *P. serotina*, *Fagus sylvatica*, and *Q. robur* on nutrient-poor sites, and *A. platanoides*, *Fraxinus excelsior*, *Q. robur*, *Prunus cerasifera*, *Prunus padus*, *P. serotina*, *F. alnus*, *U. minor* and *Sambucus nigra* on nutrient-rich sites. *Robinia pseudoacacia* biomass was positively correlated with *S. aucuparia*, *R. pseudoacacia*, and *S. nigra* on nutrient-poor sites, and *R. pseudoacacia*, *A. platanoides*, *Acer campestre*, *Acer pseudoplatanus*, *U. minor*, *F. alnus*, *P. serotina*, *P. cerasifera*, *C. avellana*, *Euonymus europaeus*, and *S. nigra* on nutrient-rich sites (Fig. 5).

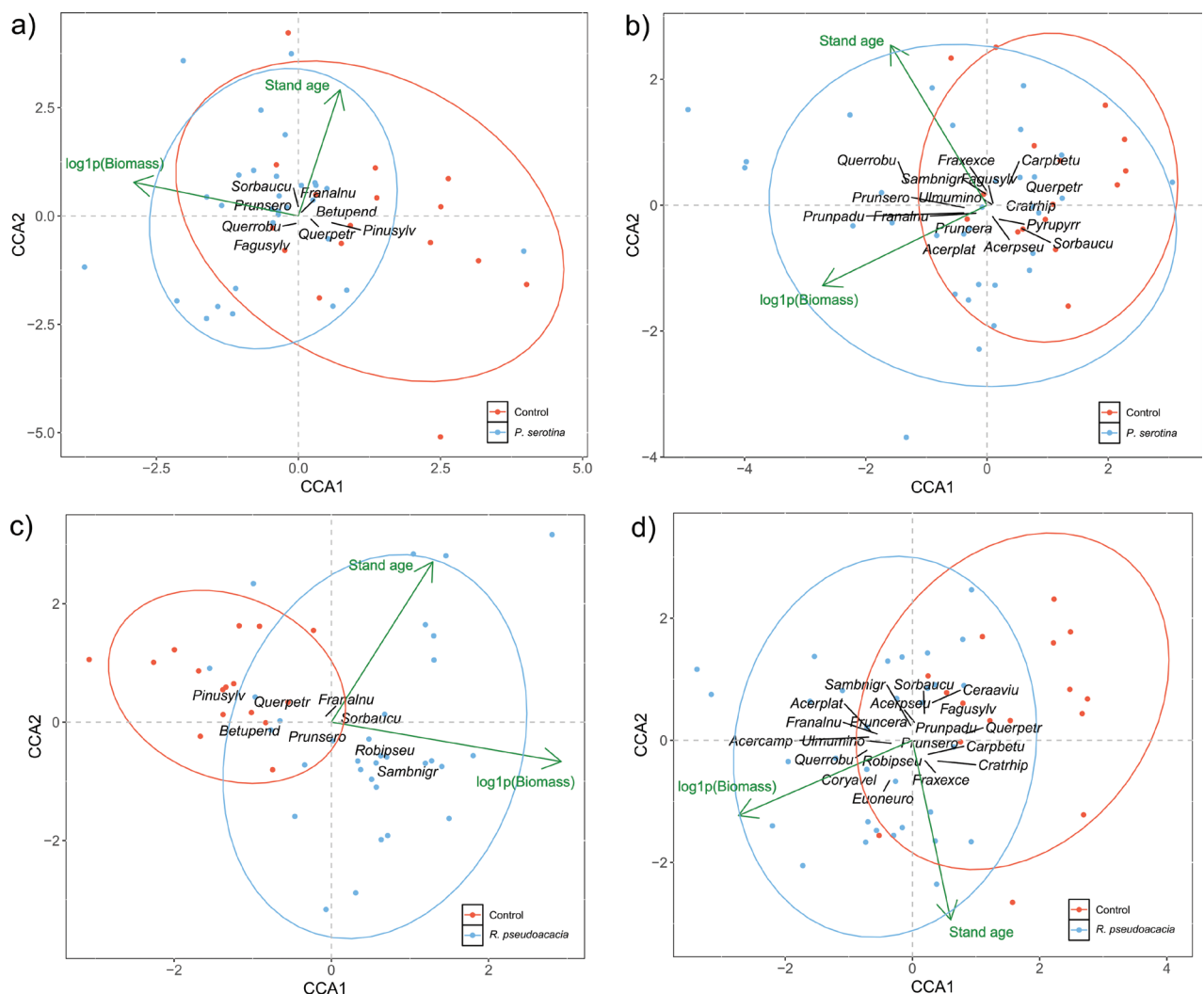


Figure 5. Canonical Correspondence Analysis (CCA) for **a** nutrient-poor sites with *P. serotina* ($n = 47$ plots) **b** nutrient-rich sites with *P. serotina* ($n = 48$ plots) **c** nutrient-poor sites with *R. pseudoacacia* ($n = 48$ plots) **d** nutrient-rich sites with *R. pseudoacacia* ($n = 48$ plots). Species with a frequency $> 20\%$ are labeled. Green arrows and green labels represent environmental variables. Red dots = control plots, light blue dots = plots with *P. serotina* or *R. pseudoacacia*. Abbreviations: log1p(Biomass) — natural logarithm of invader aboveground biomass.

Table 2. Results of permutation-based ANOVA-like test (999 iterations) of constraints significance for CCA. Abbreviations: log1p(Biomass) — natural logarithm of invader aboveground biomass.

	Df	χ^2	F	Pr(>F)
<i>P. serotina</i> nutrient-poor sites (n = 47 plots)				
log1p(Biomass)	1	0.0791	1.6678	0.038
Stand age	1	0.0552	1.1635	0.376
Residual	44	2.0870		
<i>P. serotina</i> nutrient-rich sites (n = 48 plots)				
log1p(Biomass)	1	0.1446	1.9633	0.005
Stand age	1	0.1048	1.4232	0.119
Residual	45	3.3133		
<i>R. pseudoacacia</i> nutrient-poor sites (n = 48 plots)				
log1p(Biomass)	1	0.2372	3.1398	0.001
Stand age	1	0.1725	2.2833	0.004
Residual	45	3.3991		
<i>R. pseudoacacia</i> nutrient-rich sites (n = 48 plots)				
log1p(Biomass)	1	0.1753	2.3626	0.001
Stand age	1	0.1201	1.6184	0.034
Residual	45	3.3382		

Abbreviations: **Df** – degrees of freedom; χ^2 – Chi-squared statistics; **F** – F-statistics; **Pr(>F)** – p-values.

Threshold Indicator Taxa Analysis

For *P. serotina*, we observed similar trends on both nutrient-poor and nutrient-rich sites (Fig. 6a, b, Suppl. material 1: tables S5, S6). The analysis revealed that *Q. petraea* saplings density declined with increasing *P. serotina* biomass and the opposite trend for *P. serotina* saplings. In the stands with *R. pseudoacacia*, more species revealed any response (Fig. 6c, d, Suppl. material 1: tables S7, S8). The decliners were *Pinus sylvestris*, *Q. petraea*, and *B. pendula* on nutrient-poor sites and *Q. petraea* on nutrient-rich sites. On the nutrient-poor sites *S. nigra*, *P. padus*, *A. platanoides*, and *R. pseudoacacia* increased their saplings density with increasing *R. pseudoacacia* biomass. On the nutrient-rich sites *A. platanoides*, *Q. robur*, and *R. pseudoacacia* increased their saplings density with increasing *R. pseudoacacia* biomass (Fig. 6c, d, Suppl. material 1: tables S7, S8).

Generalized linear mixed-effect models (GLMMs)

Prunus serotina on nutrient-poor sites

The density of all alien species saplings (without *P. serotina*) decreased from 2.3 ± 1.3 in control plots to 0.2 ± 1.3 in stands with 16 Mg ha^{-1} of *P. serotina*. Three species decreased their density with increasing *P. serotina* aboveground biomass. We found the highest effect size for *Q. petraea*. The number of individuals decreased from 24.3 ± 0.3 in control plots to 9.9 ± 0.4 in stands with 16 Mg ha^{-1} of *P. serotina*. *Pinus sylvestris* and *Q. robur* also reacted negatively but with smaller effect sizes. *Pinus sylvestris* individuals decreased from 1.7 ± 1.2 in control plots to 0.4 ± 1.2 in stands with 16 Mg ha^{-1} of *P. serotina*. *Quercus robur* individuals decreased from 0.7 ± 2.2 in control plots to 0.0 ± 2.3 in stands with 16 Mg ha^{-1} of *P. serotina*. Three species increased their density with increasing *P. serotina* aboveground biomass. *Prunus serotina* regenerated the best. The number of its individuals increased from

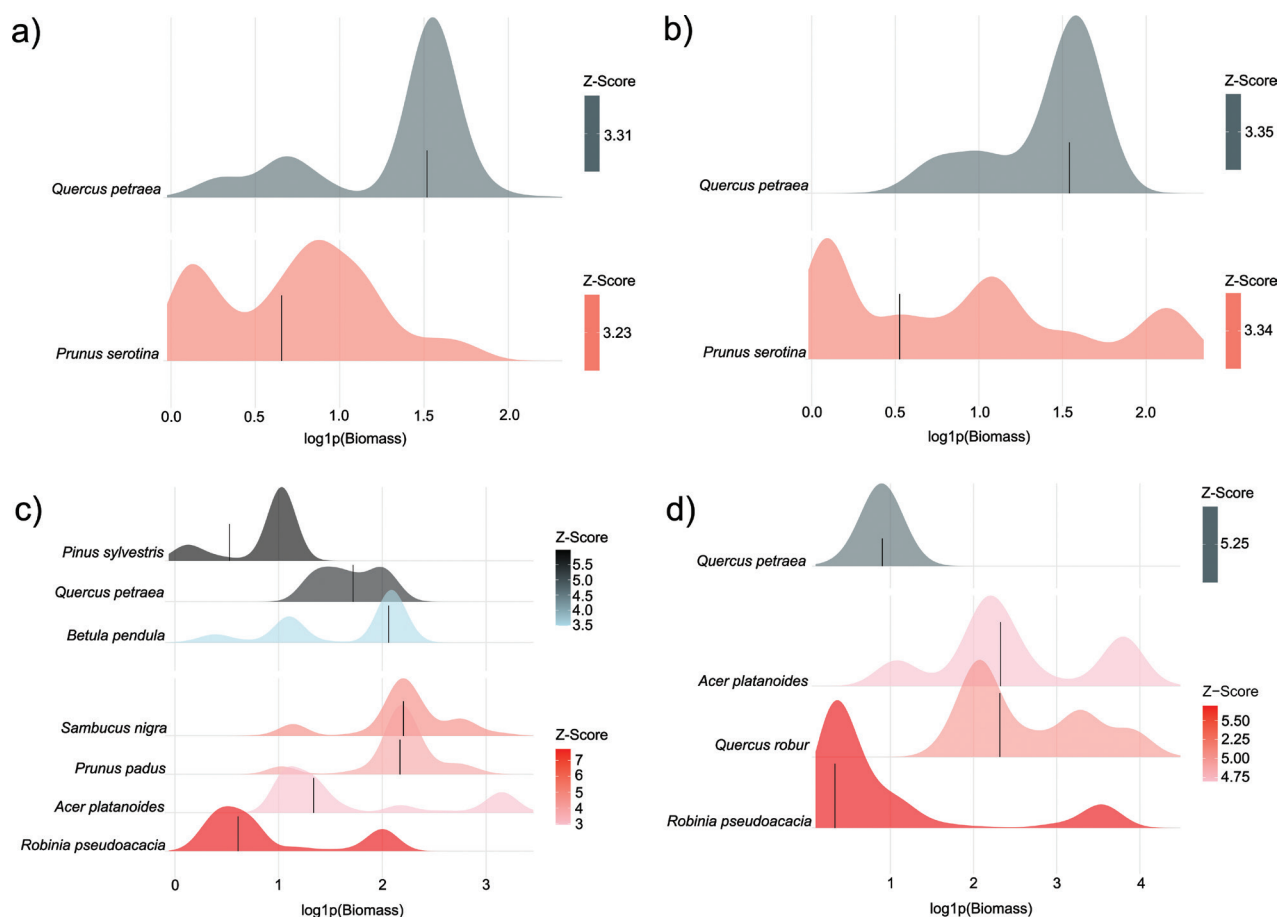


Figure 6. Results of Threshold Indicator Taxa Analysis (see Methods section for interpretation of the graph) for **a** nutrient-poor sites with *P. serotina* (n = 47 plots) **b** nutrient-rich sites with *P. serotina* (n = 48 plots) **c** nutrient-poor sites with *R. pseudoacacia* (n = 48 plots) **d** nutrient-rich sites with *R. pseudoacacia* (n = 48 plots). Grey/blue density estimators represent species responding negatively to invader biomass gradient (decliners) while red color – positively (increasers). We included here only responses for species that were both reliable (reliability ≥ 0.95) and pure (purity ≥ 0.95). For statistics of all species see Suppl. material 1: tables S5–S8.

10.0 ± 0.3 in control plots to 275.6 ± 0.3 in stands with 16 Mg ha^{-1} of *P. serotina*. The other increasers were *S. aucuparia* and *B. pendula* (Table 3, Suppl. material 1: table S9, Fig. 7). We found an increasing number of *P. serotina* and *Q. petraea* seedlings and decreasing number of *P. sylvestris* seedlings with an increase in *P. serotina* aboveground biomass (Table 3, Suppl. material 1: table S14, Fig. 8).

Prunus serotina on nutrient-rich sites

Saplings of two species decreased their density with increasing *P. serotina* aboveground biomass. We observed the highest effect size for *Q. petraea*. The number of individuals decreased from 3.5 ± 2.0 in control plots to 0.2 ± 2.0 in stands with 28 Mg ha^{-1} of *P. serotina*. *Carpinus betulus* was the second decliner, but with a lower effect size. The number of individuals decreased from 1.3 ± 0.8 in control plots to 0.6 ± 0.8 in stands with 28 Mg ha^{-1} of *P. serotina*. Four species increased their density with increasing *P. serotina* aboveground biomass. Similarly to the nutrient-poor sites, *P. serotina* regenerated the best. The number of individuals increased from 2.5 ± 0.6 in control plots to 90.0 ± 0.6 in stands with 28 Mg ha^{-1} of *P. serotina*. The other increasers, but with lower effect sizes, were *F. excelsior*, *U. minor*, and *P. padus* (Table 4, Suppl. material 1: table S10,

Fig. 9). We found an increasing number of *P. serotina* and *A. pseudoplatanus* seedlings with an increase in *P. serotina* aboveground biomass (Table 4, Suppl. material 1: table S15, Fig. 10).

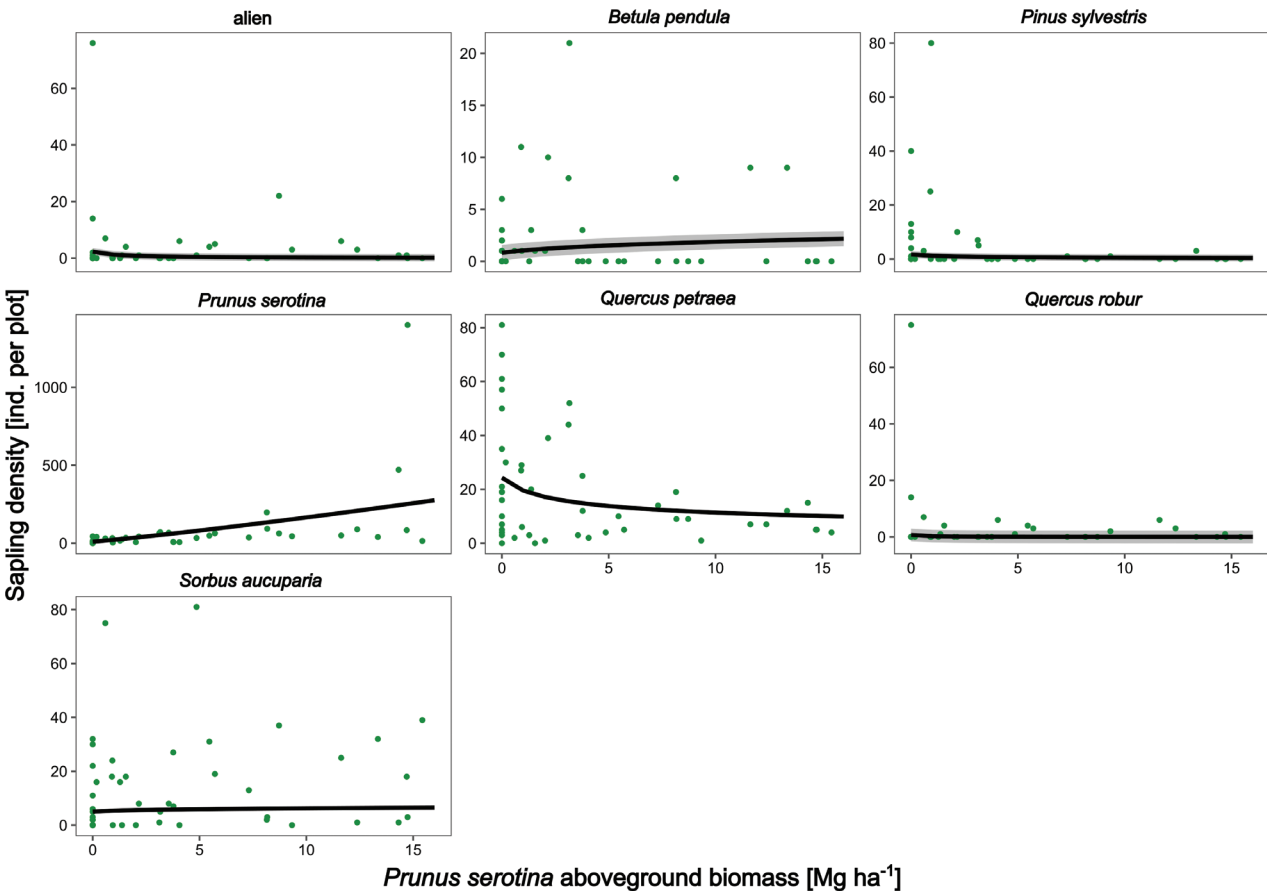


Figure 7. Generalized linear mixed-effect models for sapling density [ind. per plot] of particular species depending on *Prunus serotina* aboveground biomass [Mg ha⁻¹] in nutrient-poor sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses ± standard error, alien — density of all alien species saplings excluding *P. serotina*.

Table 3. Predictions of natural regeneration density [ind. per plot] along *P. serotina* aboveground biomass gradient on the nutrient-poor sites, estimated using Generalized Linear Mixed-effect Models. The predicted values are marginal responses from models (Suppl. material 1: tables S9, S14), assuming constant (mean) stand age and excluding random effects.

Species	<i>P. serotina</i> aboveground biomass [Mg ha ⁻¹]									
	0		2		6		10		16	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
SAPLINGS										
All alien species (without <i>P. serotina</i>)	2.3	1.3	0.8	1.3	0.4	1.3	0.3	1.3	0.2	1.3
<i>Quercus petraea</i>	24.3	0.3	17.2	0.3	13.1	0.3	11.4	0.3	9.9	0.4
<i>Quercus robur</i>	0.7	2.2	0.1	2.2	0.0	2.2	0.0	2.2	0.0	2.3
<i>Pinus sylvestris</i>	1.7	1.2	1.0	1.2	0.6	1.2	0.5	1.2	0.4	1.2
<i>Prunus serotina</i>	10.0	0.3	36.2	0.2	97.6	0.2	165.6	0.2	275.6	0.3
<i>Sorbus aucuparia</i>	5.1	0.7	5.6	0.7	6.0	0.7	6.3	0.7	6.5	0.7
<i>Betula pendula</i>	0.8	0.8	1.2	0.7	1.6	0.7	1.9	0.7	2.2	0.7
SEEDLINGS										
<i>Prunus serotina</i>	1.2	1.1	3.6	1.1	8.2	1.1	12.9	1.1	19.7	1.1
<i>Pinus sylvestris</i>	1.0	0.6	0.7	0.6	0.5	0.6	0.4	0.7	0.3	0.7
<i>Quercus petraea</i>	0.1	1.0	0.2	0.9	0.4	0.9	0.7	0.9	1.0	0.9

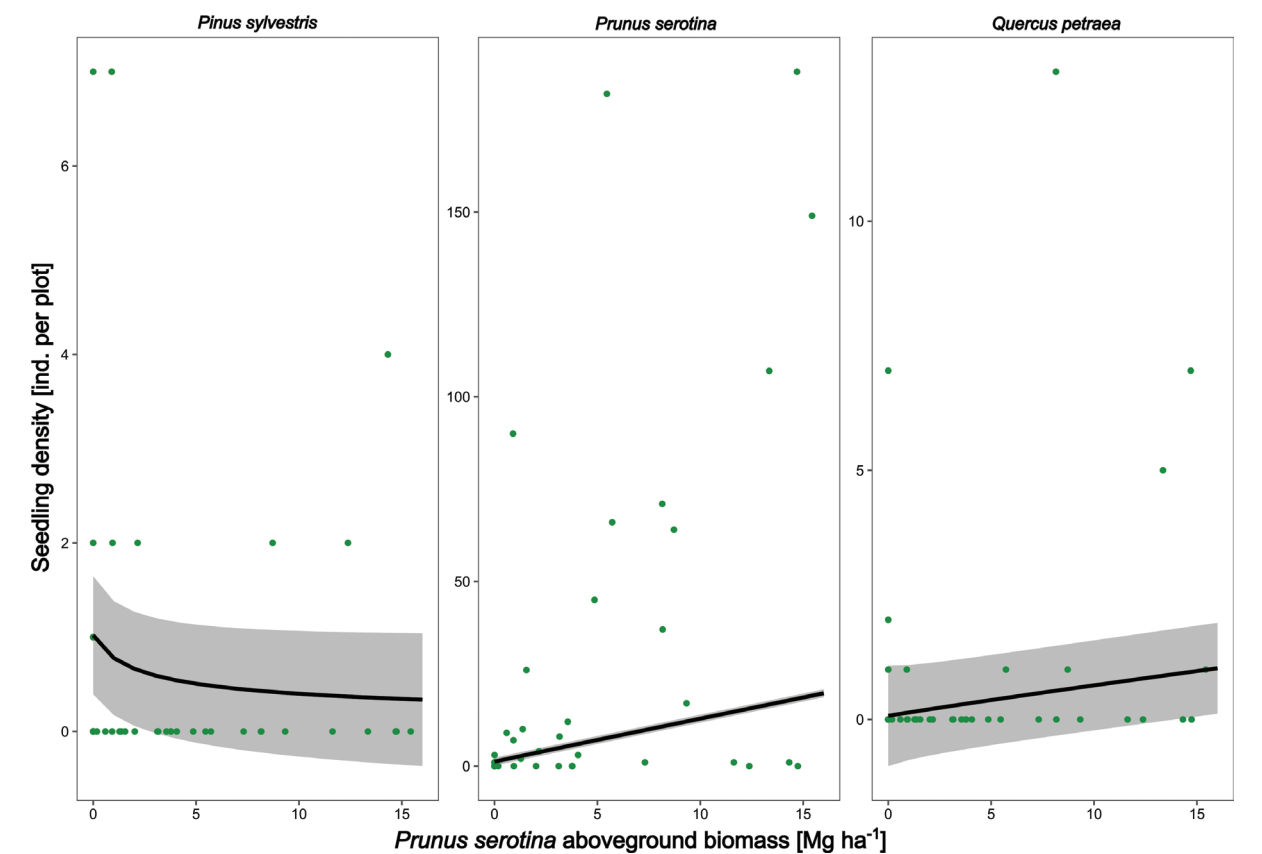


Figure 8. Generalized linear mixed-effect models for seedling density [ind. per plot] of particular species depending on *Prunus serotina* aboveground biomass [Mg ha⁻¹] in nutrient-poor sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses ± standard error.

Table 4. Predictions of natural regeneration density [ind. per plot] along *P. serotina* aboveground biomass gradient on the nutrient-rich sites, estimated using Generalized Linear Mixed-effect Models. The predicted values are marginal responses from models (Suppl. material 1: tables S10, S15), assuming constant (mean) stand age and excluding random effects.

Species	<i>P. serotina</i> aboveground biomass [Mg ha ⁻¹]									
	0		4		10		18		28	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
SAPLINGS										
<i>Quercus petraea</i>	3.5	2.0	0.9	2.0	0.5	2.0	0.3	2.0	0.2	2.0
<i>Carpinus betulus</i>	1.3	0.8	0.9	0.8	0.7	0.8	0.6	0.8	0.6	0.8
<i>Prunus serotina</i>	2.5	0.6	13.7	0.6	31.9	0.6	57.2	0.6	90.0	0.6
<i>Fraxinus excelsior</i>	2.6	0.8	10.4	0.8	20.5	0.8	32.8	0.8	47.2	0.8
<i>Ulmus minor</i>	0.2	1.0	0.4	1.0	0.6	1.0	0.8	1.0	1.1	1.0
<i>Prunus padus</i>	0.3	1.0	1.7	1.0	3.9	1.0	6.9	1.0	10.9	1.0
SEEDLINGS										
<i>Prunus serotina</i>	0.3	1.0	1.7	1.0	3.9	1.0	6.9	1.0	10.8	1.0
<i>Acer pseudoplatanus</i>	0.3	1.0	1.7	1.0	3.9	1.0	6.9	1.0	10.8	1.0

Robinia pseudoacacia on nutrient-poor sites

The number of all alien species saplings (without *R. pseudoacacia*) increased from 3.2 ± 0.3 in control plots to 21.3 ± 0.3 in stands with 116 Mg ha^{-1} of *R. pseudoacacia*. The number of *S. aucuparia* individuals increased from 7.8 ± 0.3 in control plots to 13.3 ± 0.3 in stands with 116 Mg ha^{-1} of *R. pseudoacacia*. The number of *Q. petraea* individuals decreased from 12.5 ± 0.5 in control plots to 0.9 ± 0.5 in stands with

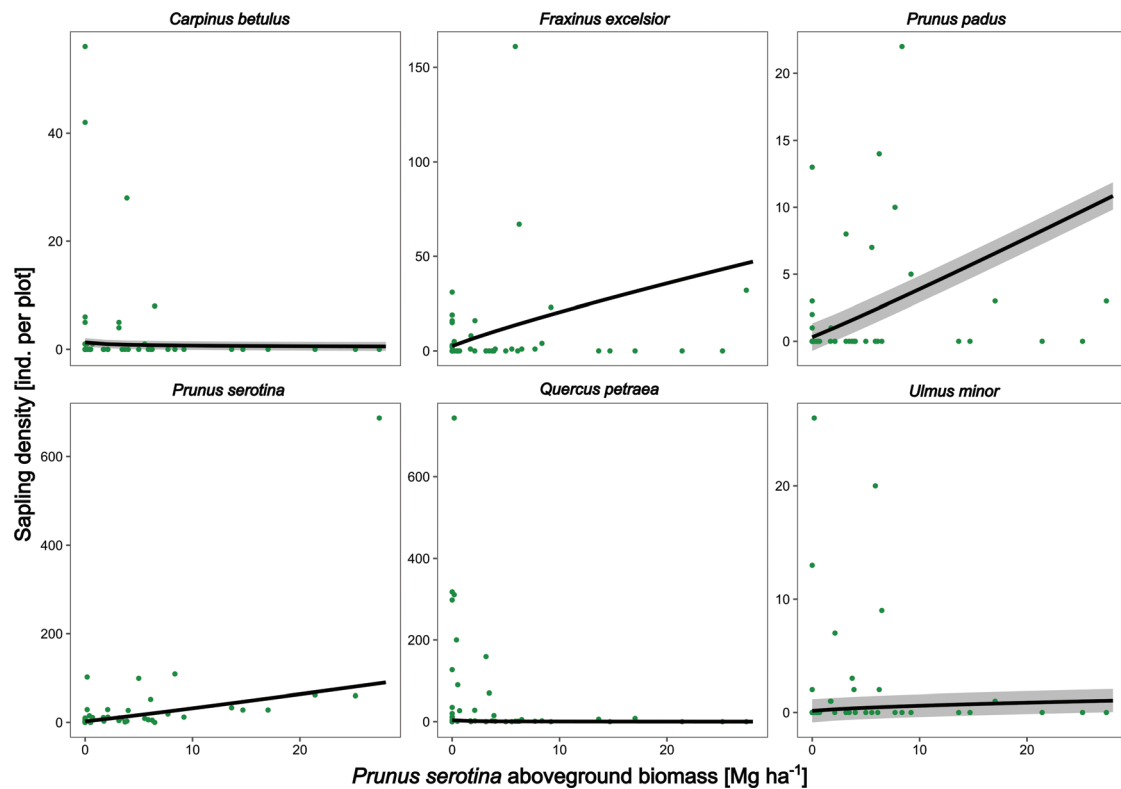


Figure 9. Generalized linear mixed-effect models for sapling density [ind. per plot] of particular species depending on *Prunus serotina* aboveground biomass [Mg ha^{-1}] in nutrient-rich sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses \pm standard error.

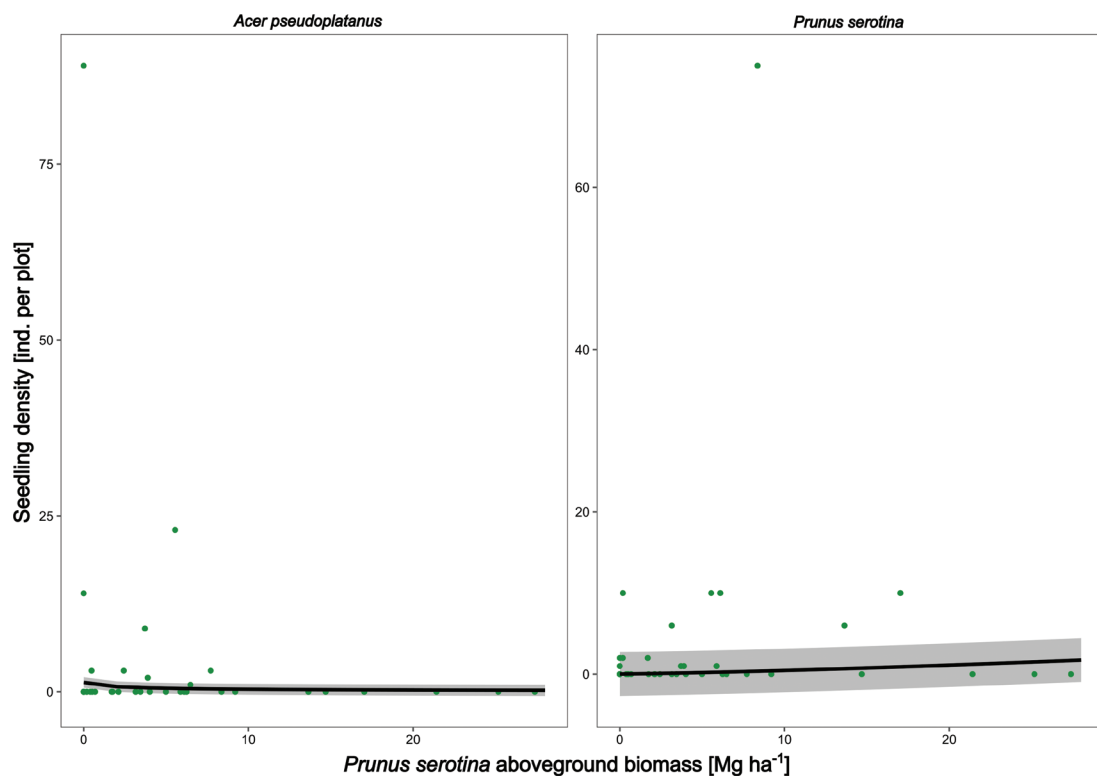


Figure 10. Generalized linear mixed-effect models for seedling density [ind. per plot] of particular species depending on *Prunus serotina* aboveground biomass [Mg ha^{-1}] in nutrient-rich sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses \pm standard error.

116 Mg ha⁻¹ of *R. pseudoacacia*. For *R. pseudoacacia* saplings, we found significant results for the relationship with aboveground biomass for the zero-inflation model, showing that a higher quantity of *R. pseudoacacia* in the stand was negatively correlated with *R. pseudoacacia* regeneration (Estimate = -2.1411, $p < 0.001$) (Table 5, Suppl. material 1: table S11, Fig. 11). We found an increasing number of *R. pseudoacacia* seedlings and a decreasing number of *P. sylvestris* seedlings with an increase in *R. pseudoacacia* aboveground biomass (Table 5, Suppl. material 1: table S16, Fig. 12).

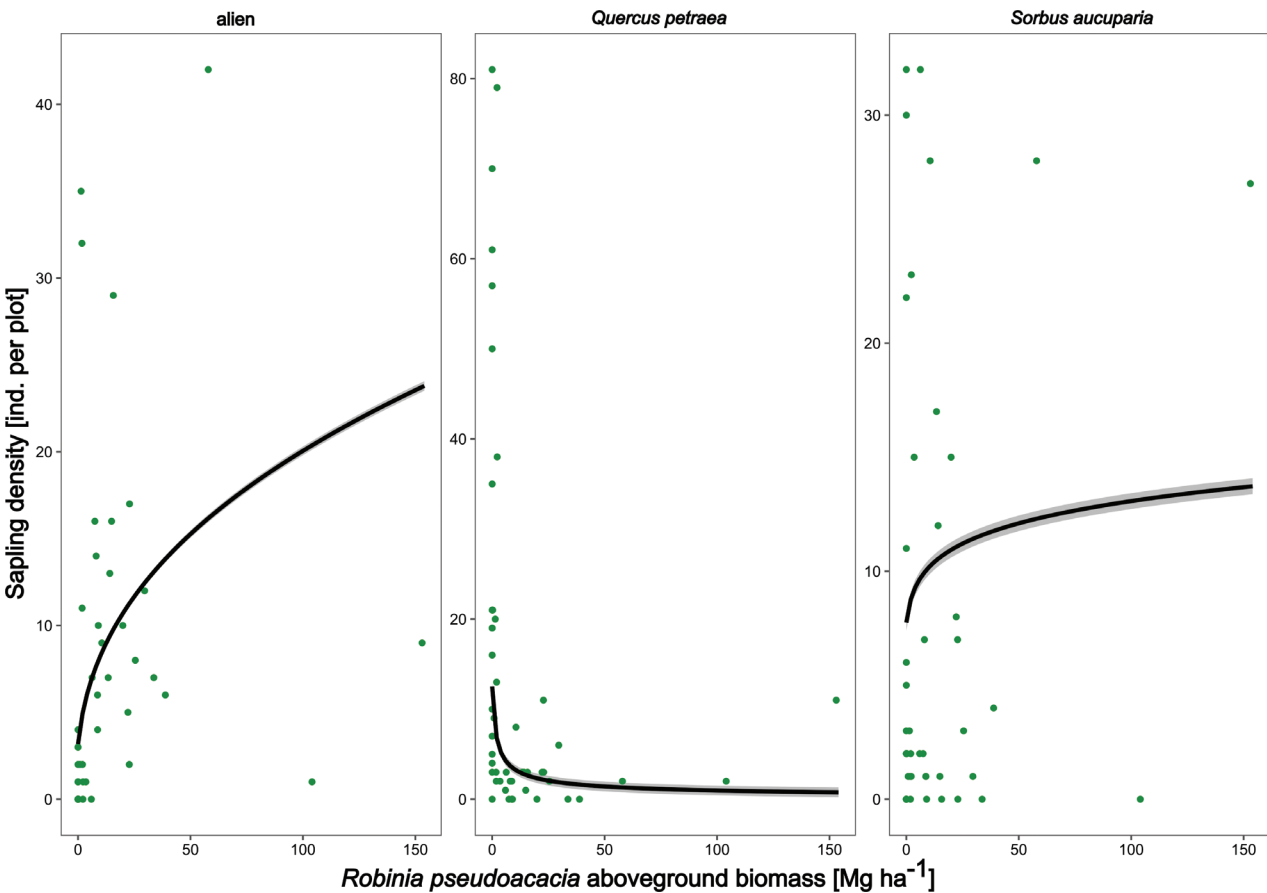


Figure 11. Generalized linear mixed-effect models for sapling density [ind. per plot] of particular species depending on *R. pseudoacacia* aboveground biomass [Mg ha⁻¹] in nutrient-poor sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses \pm standard error, alien — density of all alien species saplings excluding *R. pseudoacacia*.

Table 5. Predictions of natural regeneration density [ind. per plot] along *R. pseudoacacia* aboveground biomass gradient on the nutrient-poor sites, estimated using Generalized Linear Mixed-effect Models. The predicted values are marginal responses from models (Suppl. material 1: tables S11, S16), assuming constant (mean) stand age and excluding random effects.

Species	<i>R. pseudoacacia</i> aboveground biomass [Mg ha ⁻¹]									
	0		20		38		78		116	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
SAPLINGS										
All alien species (without <i>R. pseudoacacia</i>)	3.2	0.3	10.7	0.2	13.7	0.2	18.2	0.2	21.3	0.3
<i>Quercus petraea</i>	12.5	0.5	2.3	0.5	1.7	0.5	1.1	0.5	0.9	0.5
<i>Sorbus aucuparia</i>	7.8	0.3	10.9	0.3	11.7	0.3	12.7	0.3	13.3	0.3
SEEDLINGS										
<i>Robinia pseudoacacia</i>	0.0	2.0	0.1	2.0	0.2	2.0	0.4	2.0	0.5	2.0
<i>Pinus sylvestris</i>	0.2	1.2	0.1	1.2	0.1	1.2	0.1	1.3	0.1	1.3

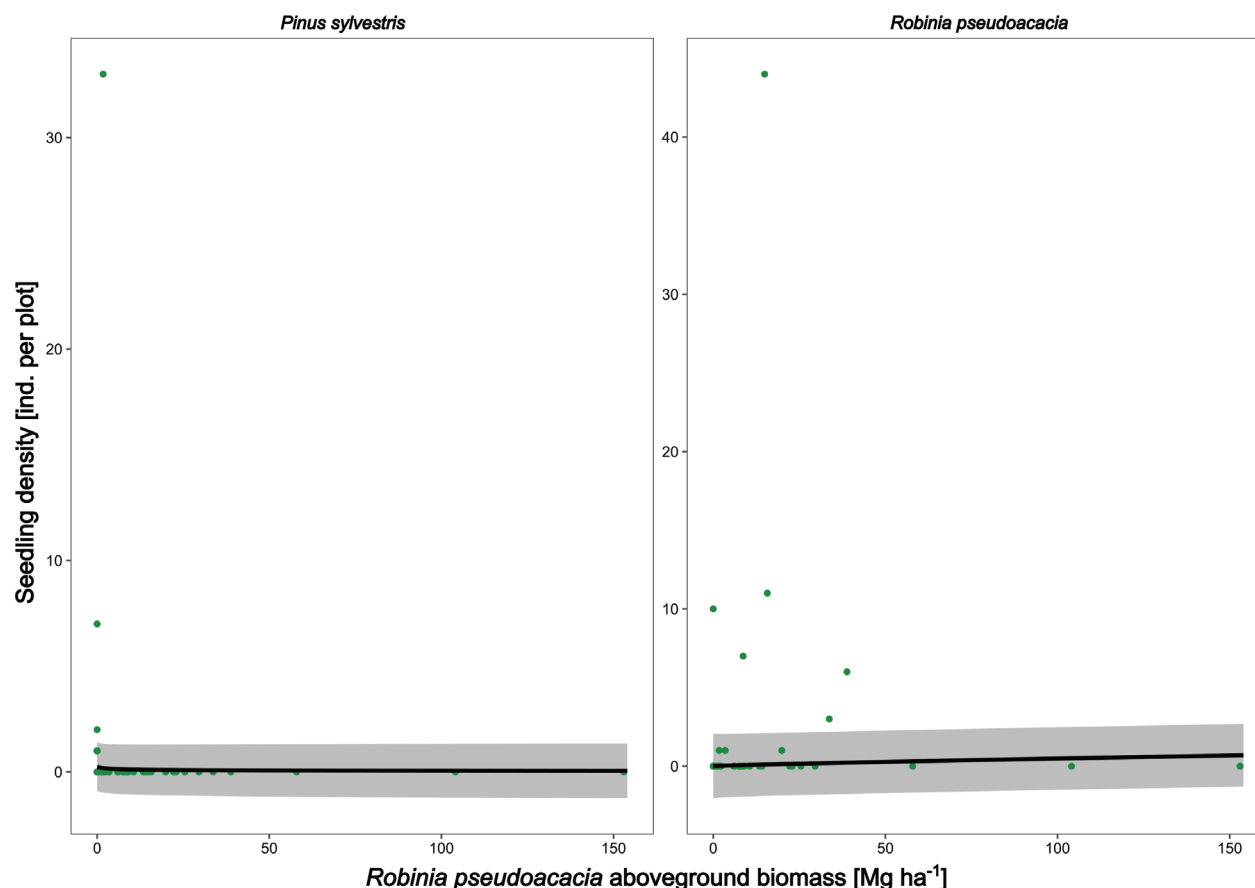


Figure 12. Generalized linear mixed-effect models for seedling density [ind. per plot] of particular species depending on *R. pseudoacacia* aboveground biomass [Mg ha^{-1}] in nutrient-poor sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses \pm standard error.

Robinia pseudoacacia on nutrient-rich sites

The density of all alien species saplings (excluding *R. pseudoacacia*) increased from 2.8 ± 0.4 in control plots to 13.0 ± 0.4 in stands with 208 Mg ha^{-1} of *R. pseudoacacia*. Four species decreased their density, and 13 species increased their density with increasing *R. pseudoacacia* aboveground biomass. Mainly forest-forming species like *Q. petraea* and *F. sylvatica* decreased the density of saplings, while species occurring usually as an admixture in the stands (all native *Acer* spp., *F. excelsior*, and *U. minor*) and shrubs (*S. nigra*, *C. avellana*, *E. europaeus*, *Crataegus rhipidophylla*, and *F. alnus*) increased their saplings density with increasing *R. pseudoacacia* biomass. We found low negative effects of increasing *R. pseudoacacia* biomass on the saplings of *C. avium* and invasive *P. cerasifera*. Some of the species reached quite high effect sizes. The number of *Q. petraea* individuals decreased from 8.8 ± 0.8 in control plots to 0.1 ± 0.9 in stands with 208 Mg ha^{-1} of *R. pseudoacacia*. The number of *A. pseudoplatanus* individuals increased from 9.5 ± 0.6 in control plots to 36.9 ± 0.6 in stands with 208 Mg ha^{-1} of *R. pseudoacacia*. The number of *F. excelsior* individuals increased from 15.0 ± 0.7 in control plots to 36.8 ± 0.8 in stands with 208 Mg ha^{-1} of *R. pseudoacacia*. The number of *S. nigra* individuals increased from 1.8 ± 0.6 in control plots to 10.9 ± 0.6 in stands with 208 Mg ha^{-1} of *R. pseudoacacia* (Table 6, Suppl. material 1: table S12, Fig. 13). We found a decreasing number of *Q. petraea* and *A. pseudoplatanus* seedlings with an increase in *R. pseudoacacia* aboveground biomass (Table 6, Suppl. material 1: table S17, Fig. 14).

Table 6. Predictions of natural regeneration density [ind. per plot] along *R. pseudoacacia* aboveground biomass gradient on the nutrient-rich sites, estimated using Generalized Linear Mixed-effect Models. The predicted values are marginal responses from models (Suppl. material 1: tables S12, S17), assuming constant (mean) stand age and excluding random effects.

Species	<i>R. pseudoacacia</i> aboveground biomass [Mg ha ⁻¹]									
	0		34		70		138		208	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
SAPLINGS										
All alien species (without <i>R. pseudoacacia</i>)	2.8	0.4	7.8	0.3	9.5	0.3	11.6	0.4	13.0	0.4
<i>Quercus petraea</i>	8.8	0.8	0.4	0.8	0.2	0.8	0.1	0.9	0.1	0.9
<i>Fagus sylvatica</i>	1.0	0.6	0.2	0.6	0.2	0.7	0.1	0.8	0.1	0.8
<i>Cerasus avium</i>	0.5	1.1	0.0	1.3	0.0	1.4	0.0	1.5	0.0	1.5
<i>Prunus cerasifera</i>	0.4	2.6	0.1	2.6	0.1	2.6	0.1	2.6	0.1	2.6
<i>Robinia pseudoacacia</i>	1.1	0.7	2.7	0.7	3.1	0.7	3.7	0.7	4.1	0.7
<i>Prunus serotina</i>	0.6	0.6	2.8	0.6	3.8	0.6	5.1	0.6	6.1	0.6
<i>Quercus robur</i>	0.0	1.5	0.2	1.4	0.2	1.4	0.3	1.5	0.3	1.5
<i>Acer pseudoplatanus</i>	9.5	0.6	23.4	0.6	28.0	0.6	33.3	0.6	36.9	0.6
<i>Acer platanoides</i>	0.1	0.9	2.0	0.9	3.6	0.9	6.3	0.9	8.9	0.9
<i>Acer campestre</i>	0.1	2.4	0.4	2.4	0.5	2.4	0.7	2.4	0.9	2.4
<i>Fraxinus excelsior</i>	15.0	0.7	27.3	0.7	30.7	0.7	34.4	0.8	36.8	0.8
<i>Ulmus minor</i>	0.4	1.0	1.3	1.0	1.7	1.0	2.1	1.0	2.5	1.0
<i>Sambucus nigra</i>	1.8	0.6	6.0	0.6	7.6	0.6	9.5	0.6	10.9	0.6
<i>Corylus avellana</i>	0.2	0.5	0.9	0.3	1.2	0.4	1.6	0.4	1.9	0.4
<i>Euonymus europaeus</i>	0.0	1.1	0.4	1.1	0.5	1.0	0.8	1.1	1.1	1.1
<i>Crataegus rhipidophylla</i>	0.1	1.1	0.3	1.0	0.4	1.0	0.6	1.0	0.7	1.0
<i>Frangula alnus</i>	0.1	1.5	0.3	1.5	0.4	1.5	0.4	1.5	0.5	1.5
SEEDLINGS										
<i>Quercus petraea</i>	0.1	1.8	0.0	1.8	0.0	1.8	0.0	1.9	0.0	1.9
<i>Acer pseudoplatanus</i>	0.6	1.5	0.1	1.5	0.0	1.5	0.0	1.6	0.0	1.6

Comparison of the methods

We used three different types of analyses, and in almost all cases we reached consistent results (Table 7). Among three tested methods we found the most consistent relationships for *Q. petraea* in all variants and *P. serotina* in plots with *P. serotina*. According to the Threshold Indicator Taxa Analysis, on the nutrient-poor sites with *P. serotina*, *B. pendula* were correlated negatively with the invader biomass, but according to the GLMMs positively. We found a contrast pattern in the case of *R. pseudoacacia* regeneration in stands with *R. pseudoacacia*, as TITAN2 suggested a positive relationship, but the model suggested a negative (but only for the zero-inflation component).

Discussion

General patterns

Observational studies on the impact of invasive species on various ecosystems, including forests, should not be considered as a simple causation based on observed correlations. Ecosystems are very complex and each of their elements is simultaneously affected by various factors. Impact assessment should be multidimensional and a systemic approach. In our plots we observed different densities of saplings and seedlings of individual species. We refer to individual hypotheses in the

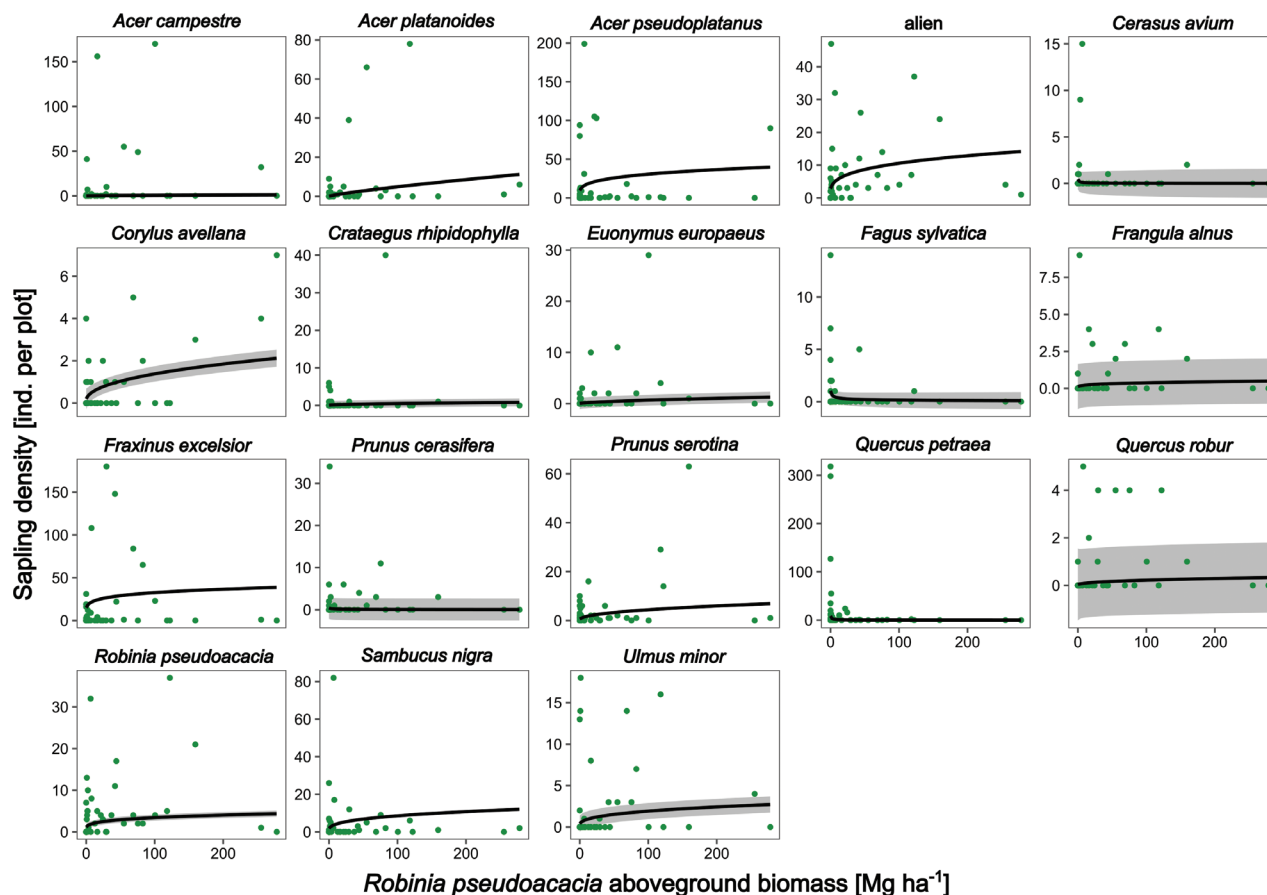


Figure 13. Generalized linear mixed-effect models for sapling density [ind. per plot] of particular species depending on *R. pseudoacacia* aboveground biomass [Mg ha^{-1}] in nutrient-rich sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses \pm standard error, alien — density of all alien species saplings excluding *R. pseudoacacia*.

following sections of the discussion. The relationship between natural regeneration density and biomass of *P. serotina* and *R. pseudoacacia* can be both positive or negative, and this is in line with recent studies showing that results depend on the environmental context (Sapsford et al. 2020; Catford et al. 2022) and the reference ecosystem used for comparison (Sádlo et al. 2017; Medvecká et al. 2018; Dyderski and Jagodziński 2021b). Our study improves the knowledge about the relationship between different invasive tree biomasses and ecosystem services.

Species-specific patterns

We found different relationships between particular species natural regeneration densities and *R. pseudoacacia* and *P. serotina* biomasses (H1, H2). We confirm both the first (H1) and second (H2) hypotheses. The biomass of *R. pseudoacacia* was correlated with the density of natural regeneration more than *P. serotina* (H1). We also confirm the second hypothesis, as individual natural regeneration species showed different patterns of density. Some showed a decrease in density with the biomass increase of invaders, others showed opposite trends. Differences between individual species were seen in the number of individuals in each quantity of the invasive species, the shape (more linear or exponential), and the slope of the curves in the models. Similarly, in TITAN2, we found differences in the number of species and shapes of ridges (H2). Some natural regeneration species revealed

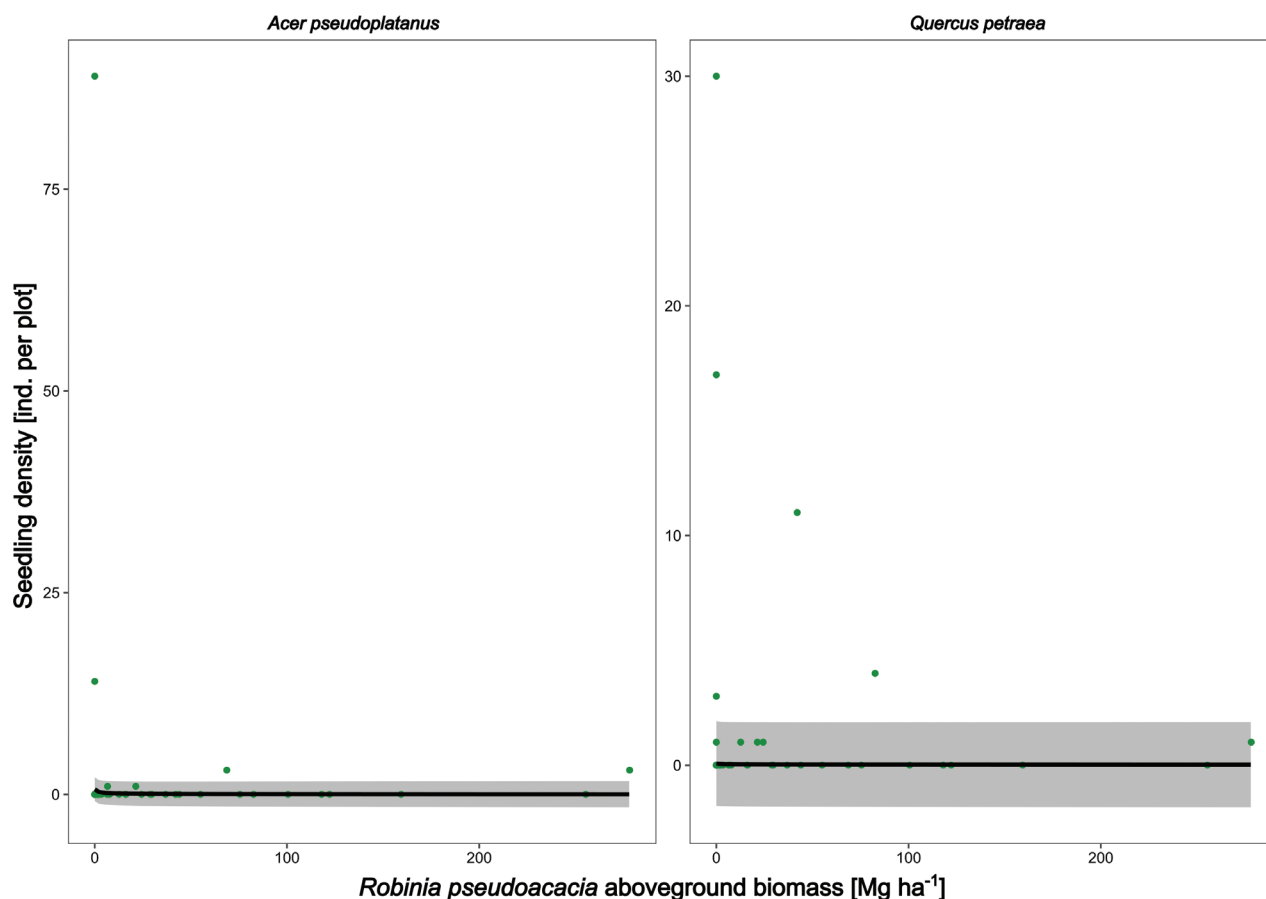


Figure 14. Generalized linear mixed-effect models for seedling density [ind. per plot] of particular species depending on *R. pseudoacacia* aboveground biomass [Mg ha⁻¹] in nutrient-rich sites. Dark green dots — observed values, black line — marginal responses, grey area — marginal responses \pm standard error.

positive relationships with invader biomass (mostly *P. serotina* in the stand with *P. serotina*) and some negative (*Q. petraea*, *P. sylvestris*). Those differences between particular species relationship with *R. pseudoacacia* or *P. serotina* should be mostly connected with different light or nutrient requirements of particular sapling and seedling species. As the transformations of both studied neophytes changed along their biomasses, also the density of particular natural regeneration species should change more intensively. There are visible trends connected with the ecological niches of particular trees, but they should be interpreted with caution. More shade-tolerant and nitrophilous species increased their abundances with invader biomass increasing, e.g., *F. excelsior* or *Acer* spp. In contrast, light-demanding and acidophilous *P. sylvestris* decreased its abundance or the abundance remained unchanged. Increasing biomass of either *P. serotina* or *R. pseudoacacia* led to reduced light availability on the forest floor and higher nutrient content in the soil (Rice et al. 2004; Dyderski and Jagodziński 2019; Engel et al. 2024). Those transformations did not support *P. sylvestris* regarding natural regeneration growth, in both saplings and seedlings. Lázaro-Lobo et al. (2021) also mentioned that the response of a particular species' natural regeneration depends on their functional traits, and the competition between invasive tree species with desirable species depends on their niche spacing. There are also visible differences in the number of significant relationships between sapling species densities and *R. pseudoacacia* or *P. serotina* biomasses (H1). Focusing only on GLMMs (Table 7), in nutrient-rich habitats

Table 7. Summary of saplings species' responses to invasive trees according to different analyses. CCA based on species with frequency > 20%, TITAN2 based on species with purity and reliability >= 0.95. GLMMs based on statistically significant results for the effect of invader aboveground biomass.

	<i>Prunus serotina</i>						<i>Robinia pseudoacacia</i>					
	Poor sites			Rich sites			Poor sites			Rich sites		
	C	T	M	C	T	M	C	T	M	C	T	M
<i>Acer campestre</i>										+		+
<i>Acer platanoides</i>				+				+		+	+	+
<i>Acer pseudoplatanus</i>				—						+		+
<i>Betula pendula</i>	—		+				—	—				
<i>Corylus avellana</i>										+		+
<i>Cerasus avium</i>										—		—
<i>Carpinus betulus</i>				—		—				—		
<i>Crataegus rhipidophylla</i>				—						—		+
<i>Euonymus europaeus</i>										+		+
<i>Frangula alnus</i>	—			+			+			+		+
<i>Fraxinus excelsior</i>				+		+				—		+
<i>Fagus sylvatica</i>	+			?						—		—
<i>Prunus cerasifera</i>				+						+		—
<i>Prunus padus</i>				+		+		+		—		
<i>Pyrus pyraister</i>				—								
<i>Prunus serotina</i>	+	+	+	+	+	+	—			—		+
<i>Pinus sylvestris</i>	—		—				—	—				
<i>Quercus petraea</i>	—	—	—	—	—	—	—	—	—	—	—	—
<i>Quercus robur</i>	+		—	+						+	+	+
<i>Robinia pseudoacacia</i>							+	+	—	+	+	+
<i>Sorbus aucuparia</i>	+		+	—			+		+	—		
<i>Sambucus nigra</i>				+			+	+		+		+
<i>Ulmus minor</i>				+		+				+		+

Abbreviations: **C** — Canonical Correspondence Analysis (CCA); **T** — Threshold Indicator Taxa Analysis (TITAN2); **M** — Generalized Linear Mixed-Effect Models (GLMMs); **+** — positive effect of invasive species; **—** — negative effect of invasive species; **?** — unclear effect of invasive species.

17 species (13 positively and four negatively) responded significantly to *R. pseudoacacia* increasing biomass and six (four positively and two negatively) to *P. serotina* increasing biomass. In nutrient-poor habitats, three species (one positively and two negatively) responded to *R. pseudoacacia* increasing biomass and six (three positively and three negatively) to *P. serotina* increasing biomass. According to GLMMs in nutrient-rich habitats (*R. pseudoacacia*: 76%, *P. serotina*: 67%), the share of the number of species reacting positively to the invader's biomass in the number of species that responded significantly positively and negatively was higher than in coniferous habitats nutrient-poor habitats (*R. pseudoacacia*: 33%, *P. serotina*: 50%; H3).

For some species, we observed some trends similar to those observed by Dyderski and Jagodziński (2020), conducted in protected forests of the nearby Wielkopolska National Park. The density of forest tree species was lower in invaded stands by both *R. pseudoacacia* and *P. serotina* than in non-invaded while shrubs and admixed trees increased their density. Our study provided a significant advance from this study, as we included invader abundance, that allows for assessment of various stages of invasion (López-Núñez et al. 2017).

Our observations regarding the negative correlation between invasive trees biomass and the natural regeneration of forest-forming species are in line with the findings of Terwei et al. (2013) and Kowarik et al. (2019). Terwei et al. (2013)

showed that in the hardwood floodplain forests, *R. pseudoacacia* in the stand was positively correlated with the density of *R. pseudoacacia* seedlings. However, *P. serotina* in the stands was positively correlated with *P. serotina* seedlings but negatively with *U. minor* seedlings. Langmaier and Lapin (2020), in their review of the impact of different invasive plant species on forest regeneration, also discussed the impact of *P. serotina* and *R. pseudoacacia*. Based on the works of other authors (e.g., Rahmonov 2009; Maringer et al. 2012; Petrášová et al. 2013; Radtke et al. 2013; Terwei et al. 2013), Langmaier and Lapin (2020) synthesized the negative impact of *R. pseudoacacia* on species e.g., *Q. petraea*, *Q. robur*, *P. sylvestris*, *U. minor*. In our studies, we confirmed a negative correlation of *Q. petraea* and *P. sylvestris* regeneration density with invader biomass. For *Q. robur* and *U. minor*, we obtained less obvious positive responses. *Quercus robur* was less frequent than *Q. petraea* in our plots, while for *U. minor* we obtained significant results only for nutrient-rich sites. However, it should be borne in mind that their work accounted for habitats not only from Central Europe, but also other ecoregions, e.g., Western European deciduous forests, Pannonian mixed forests, or riparian forests.

Ambiguous invasional meltdown and propagule pressure hypotheses

When invasive species arrive in new niches, they can change soil chemicals, transform light conditions, and make the ecosystem more suitable for the other alien species (Crooks 2002; Corenblit et al. 2014; Jagodziński et al. 2024). In the longer term, this may increase the negative impact on biodiversity and other ecosystem services. For the total alien species natural regeneration density, we confirm the invasional meltdown hypothesis (H4) (Simberloff and Holle 1999) only for *R. pseudoacacia*, both on the nutrient-poor and nutrient-rich sites. Since we examined the relationship between *R. pseudoacacia* and *P. serotina* and the total density of all alien species in the regeneration layer, it is impossible to find species-specific patterns related to their biology and ecology. However, for some individual species we obtained significant results. *Prunus serotina* regeneration density was higher in plots with higher *R. pseudoacacia* biomass on nutrient-rich sites. However, little is known about the possible interactions between the adult *R. pseudoacacia* and *P. serotina* natural regeneration and vice versa. The interesting fact was that invasive *P. cerasifera* abundances decreased with an increase in *R. pseudoacacia* aboveground biomass. Due to the similar biology and ecology of this species to *P. serotina* we would expect rather similar responses. Czortek et al. (2024) proved that the presence of *P. cerasifera* natural regeneration is favored by higher light availability. However, this species avoids places with a higher number of functional types, which may indicate its lower resistance to competition than *P. serotina*.

According to the propagule pressure hypothesis, the higher the propagule pressure, the more effective colonization (Lonsdale 1999; Lockwood et al. 2005; Blackburn et al. 2011). This dependence of studied species on propagule pressure was confirmed in numerous previous studies (Vanhellemont et al. 2009; Vítková et al. 2017; Dyderski and Jagodziński 2018). Trees acquire reproductive abilities late. However, they retain these abilities for a very long time. More propagule sources should increase the regeneration capacity. We confirmed this hypothesis for *P. serotina* on both nutrient-rich and nutrient-poor sites, but the predicted values of sapling and seedling densities were higher on nutrient-poor sites. For *R. pseudoacacia*, we confirmed this hypothesis for saplings on nutrient-rich sites and seedlings

on nutrient-poor sites, but the predicted densities were low. In general, in our plots *R. pseudoacacia* did not regenerate as effectively as *P. serotina*. *Robinia pseudoacacia* spreads generatively mostly by wind and therefore prefers open spaces. *Prunus serotina* seeds mostly fall directly into the soil and to a lesser degree are spread by birds (Deckers et al. 2008; Vanhellemont et al. 2009; Dylewski et al. 2017). Also, both *P. serotina* (Starfinger et al. 2003) and *R. pseudoacacia* (Bouteiller et al. 2023) easily regenerate vegetatively by root suckers.

Dependence on habitat context and biotic resistance/acceptance

Biological invasion dynamics can depend on several factors: environmental conditions, interactions between species, anthropogenic factors, and management (González-Moreno et al. 2014; Sapsford et al. 2020; Catford et al. 2022). To exclude the management context, we established study plots in forest patches without visible impacts of silvicultural treatments e.g., planting or removing trees from plots. We also placed them in similar climatic conditions. We expected a more distinct decline in the density of natural regeneration to invader quantity/biomass on nutrient-poor than on nutrient-rich sites (H3) (Chmura 2004; Halarewicz 2011). In general, in nutrient-rich sites, there are higher densities of native trees understory, so the competition with invasive species is stronger. This is indicated by the number of species that reached a frequency > 20%. Comparing the abundances, *P. serotina* was more successful on nutrient-poor than nutrient-rich sites. Results for *P. serotina* are connected with the Empty Niche Hypothesis (Elton 1958; Schmitt 2020). This hypothesis suggests that invasive species can successfully settle new ecosystems by occupying weakly filled or unfilled ecological niches, where native species are less common or absent. In Central Europe, nutrient-poor sites with *P. sylvestris* have lower richness and abundance of native species compared to nutrient-rich habitats. *Prunus serotina* was massively introduced on nutrient-poor sites by foresters (Starfinger et al. 2003; Engel et al. 2024; Nyssen et al. 2024). Small competition from native trees facilitated the spread of *P. serotina* to new stands and led to their dominance in these habitats. This species further spreads easily to nearby stands. Especially, on nutrient-rich sites with *R. pseudoacacia*, we can observe that many of the native tree species regeneration increased their abundance with invader biomass increasing. More shade-tolerant species showed a positive correlation with invader biomass, except for *C. betulus* on nutrient-rich sites with *P. serotina*. This finding contradicts a previous study (Dyderski and Jagodziński 2020) revealing a positive response of this species to *P. serotina* presence. However, in the cited study, this response regarded *P. sylvestris* plantations on nutrient-rich sites, thus it cannot be directly compared. Our results balance between supporting the biotic acceptance (Stohlgren et al. 2006) and biotic resistance (Elton 1958; Levine et al. 2003) hypotheses. In the case of stands with *P. serotina*, both in poor and fertile habitats, the density of its regeneration increased with the biomass of the parental trees, mainly due to the availability of propagules and dispersal mechanisms: barochory and zoochory. Nevertheless, the effect sizes were higher on the poor sites. In the case of *R. pseudoacacia* stands, we observed slightly better regeneration of this species in nutrient-rich habitats. To sum up, for *P. serotina* our results are rather in line with the biotic resistance hypothesis (Elton 1958; Levine et al. 2003), while for *R. pseudoacacia* they rather in line with biotic acceptance (Stohlgren et al. 2006). The more visible relationships between natural regeneration and

invader biomass in nutrient-poor habitats depend on specific ecological conditions, specific plant species composition, and soil fertility. Therefore, any transformation by an invasive species is more severe for the species occurring there.

Three different analyses

We had to adapt the database to the analysis guidelines. CCA was the least conservative analysis in the case of input data. CCA is also the least sensitive on extremal observations. In models, we excluded one plot for *C. betulus* saplings and two plots for *C. avium* saplings (see the rationale in the Materials and Methods section). Thanks to the use of TITAN2, we were able to detect the threshold for particular invasion levels e.g., *P. serotina* saplings reacted quickly with big abundance on even small quantity of *P. serotina* in the stands, while *Q. petraea* as a decliner was more tolerant to *P. serotina* biomass increasing. For *C. avium* omitting these records in the models did not change the trend (positive/negative) but reduced the standard error and smoothed the regression curve. In the case of *C. betulus*, removing the extreme observation changed the trend from positive to negative. The negative trend is consistent with the CCA result. The extreme observation results from the fact that there were adult *C. betulus* in the vicinity of the plot, acting as a propagule source. According to the guidelines of statistical model development, we should remove this outlier. The model after removing the outlier had a more stable distribution of residuals and a lower standard error of estimates. Even though each of the analyses we use is based on slightly different data structures and responds differently to data variability, the results we obtain are very similar. In general, consistent responses revealed by three different methods suggest that all these tools are useful in the assessment of correlations with invasive species biomass. We also found that TITAN2 resulted in the most conservative approach – for *P. serotina* and *R. pseudoacacia* on nutrient-rich sites it revealed relationships only in the cases confirmed by two other methods. For *R. pseudoacacia* on nutrient-poor sites, it revealed relationships not confirmed by two other methods only for three species.

Wider context and management implications

In the context of current trends in forestry, *P. sylvestris* is still the main species in nutrient-poor sites areas, while *Quercus* spp. is in nutrient-rich sites. Therefore, referring to habitats studied here, densities of main forest-forming species were negatively correlated with the biomass of studied invasive species, especially *Q. petraea*. *Prunus serotina* also hindered the regeneration of *P. sylvestris* in the poor sites. We found an increasing density of *Quercus robur* saplings with increasing *R. pseudoacacia* biomass in fertile habitats, but negatively correlated with *P. serotina* biomass in poor habitats. In the context of natural forests and ongoing climate change, the situation looks a bit different. Wide-scale studies predict the retreat of forest-forming tree species from Central Europe, especially *P. sylvestris*, as a response to climate change (Dyderski et al. 2018; Chakraborty et al. 2021; Wessely et al. 2024). That way, studied neophytes can enhance this negative effect by suppressing the natural regeneration of studied species. Recent management strategies propose in some cases the assimilation of invasive species with native ecosystems (Nyssen et al. 2024), also for *P. serotina* (Nyssen and Vanhellemont 2016; Engel et al. 2024) and *R. pseudoacacia* (Sádlo et al. 2017). Such a strategy is recommended especially in fertile habitats that are biotically more resistant

to the development of invasion. In assimilating studied invasive species, managers should be hypersensitive to their potential impacts on main forest-forming species. When we want to maintain or increase the number of species such as *Q. petraea* in the regeneration, we should take into account the results of our research and apply methods that will facilitate their survival. Management in fertile habitats should be adapted to a long-term management plan. If we want to mimic natural processes, the presence of *R. pseudoacacia* (based on our research) may be helpful, as it promotes species such as *F. excelsior*, *U. minor*, or *Acer* spp. On the other hand, if we want to preserve as large a *Quercus* population as possible, some human action may be necessary. In the case of *P. sylvestris* stands, we maintain the fact that if we want successful *P. sylvestris* regeneration, it is necessary to take into account the observed decreasing density of *P. sylvestris* regeneration with both invasive species studied biomass increasing and support the regeneration of *P. sylvestris*. Langmaier and Lapin (2020) summarized that there are studies that indicate that in the case of *R. pseudoacacia*, its eradication measures or adaptation of silvicultural measures are the most frequent management actions, while in the case of *P. serotina* – early detection. The latter concerns both decision-makers and the entire society, because it is easier to control biological invasions in the early stages. In turn, silvicultural treatments can be modified to promote selective cutting, and appropriately manage the closure of tree crowns and density with local reduction of invasive species combined with the promotion of species of native origin. Unfortunately, our study revealed the negative correlations between studied invasive species biomass and the *Q. petraea* natural regeneration in poor sites. Due to changing climatic conditions leading to the retreat of coniferous species, many see the potential of *Q. petraea* to replace *P. sylvestris* (Hanewinkel et al. 2013; Dyderski et al. 2025). Since *R. pseudoacacia* and *P. serotina* are very common in European forests (Wagner et al. 2017; Campagnaro et al. 2018), and are predicted to expand their range under the changing climate (Puchałka et al. 2021, 2023), we may expect the negative impact on *Q. petraea* regeneration, that will require particular attention.

Although our study focused on managed forests, certain relationships can be related to natural forests. The areas we searched had the structure of semi-natural forests, managed in a way that imitated natural processes. In the case of protected forests, it is important to monitor the presence and impact of invasive species on natural processes and prevent possible damage they may cause. Eradication of invasive trees is expensive and sometimes counter-productive or even makes the situation worse (Namura-Ochalska and Borowa 2015; Nyssen and Vanhellefont 2016; Nyssen et al. 2024). Our study should be helpful for stakeholders in making decisions about the assimilation or eradication of invasive trees in particular types of stands on particular habitats (Nyssen and Vanhellefont 2016; Sádlo et al. 2017).

Conclusions

Our study provided the first quantitative assessment of the relationships between invasive tree biomass and forest natural regeneration, along the gradient of invader biomass. Additionally, we compared patterns obtained using three different statistical approaches: ordination, Threshold Indicator Taxa Analysis, and generalized linear mixed-effects models. We confirmed that invader taxa and their biomass are important and differentiate the strength of the relationship with natural regeneration. Additionally, we observed different relationships between nutrient-rich and nutrient-poor sites. Moreover, particular tree species were differently related to invader biomass on

particular sites and with different effect sizes. The most important finding is the negative relationship of studied invasive trees on the regeneration of crucial forest-forming tree species typical of the studied habitats, such as *P. sylvestris* in poor sites and *Q. petraea* in both nutrient-poor and rich sites. In general, *P. serotina* regenerated better than *R. pseudoacacia*, especially on nutrient-poor sites. For both species, we confirmed the importance of propagule pressure, expressed by parental tree biomass. We also confirmed the invasional meltdown hypothesis for stands with *R. pseudoacacia*, as the density of all non-native saplings (excluding *R. pseudoacacia*) increased with an increase in *R. pseudoacacia*. However, we did not confirm this hypothesis for stands with *P. serotina*. We also showed that three tested statistical approaches reveal consistent results, supporting the strength of our conclusions.

The results of our study are crucial for selecting tree species that regeneration is more vulnerable to studied invaders. This knowledge can improve the prioritization of management and designation of forest patches requiring additional silvicultural treatments to maintain or initiate natural regeneration. Moreover, our results allow determining thresholds of invasive biomass at which we observed a decreasing density of natural regeneration of the main tree species. For that reason, our study is important in the managed forests promoting natural regeneration, as well as for the protected forest areas e.g., national parks or forest reserves.

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Additional information

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: SB, MKD; methodology: SB, MKD; investigation: SB, MKD; formal analysis: SB; visualization: SB; writing—original draft preparation: SB; writing—review and editing: MKD; funding acquisition: MKD.

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Data availability

All data supporting the results are archived in the figshare repository (Dyderski and Bury 2024) 10.6084/m9.figshare.26809084.

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Supplementary material 1

Supplementary information

Authors: Sebastian Bury, Marcin K. Dyderski

Data type: docx






Explanation note: This file contains supplementary details about natural regeneration species frequency, allometric models used to aboveground biomass calculation, and detailed data supporting the analyses presented in the manuscript

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Research Article

Non-consumptive effects of native, alien and invasive alien crayfish on damselfly egg life history and carry-over effects on larval physiology

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Abstract

Invasive alien (IA) predators pose significant threats to native ecosystems, often leading to profound impacts on prey species through both direct and non-consumptive effects (NCE). This study focused on the NCE of predator-induced stress from one native crayfish species, noble (*Astacus astacus*), compared to one alien danube crayfish (*Pontastacus leptodactylus*) and two IA crayfish species, signal (*Pacifastacus leniusculus*) and spinycheek crayfish (*Faxonius limosus*), on the native damselfly *Ischnura elegans*. We investigated the direct crayfish cue effect on egg traits as well as potential carry-over effects from the egg stage to the larval stage. We hypothesised that native crayfish cues would lead to more pronounced negative effects on prey traits compared to alien and IA crayfish, due to an evolutionary history of interaction and recognition of these threats. Unexpectedly, compared to native crayfish cues, alien and IA crayfish cues caused significantly higher egg mortality and prolonged developmental times, particularly cues from danube and signal crayfish, while cues from spinycheek crayfish had weaker, yet, still significant effects. Hatching synchrony was reduced and this to the same extent by the cues of all four crayfish species. Notably, cues from both alien and IA crayfish species caused significant carry-over effects, resulting in reduced larval survival, mass and fat content, which were more pronounced for danube and signal crayfish. Native crayfish cues did not induce carry-over effects, suggesting that *I. elegans* may have evolved a degree of resilience against this predator or that native crayfish produce chemical cues that do not cause a strong antipredator response. Our findings underscore the importance of considering immediate and carry over effects of crayfish on prey traits across multiple life stages, particularly in the context of biological invasions.

Key words: Carry-over effect, invasive alien species, life history, phenotypic plasticity, physiology, predator-prey interaction



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Introduction

In natural systems, ecological stressors mediated by human activity such as the introduction of invasive alien (IA) predators can drastically affect native prey populations, cascading to changes in the dynamics of native prey communities (Sih et al. 2010; Bucciarelli et al. 2019). This can be explained by the fact that native

prey lack evolutionary history with newly-introduced predators and do not recognise the predators as being dangerous (Schlaepfer et al. 2005; Anton et al. 2020). According to the “naïve prey hypothesis”, IA predators are expected to be more effective than native predators in preying upon local prey (Cox and Lima 2006). Although some studies supported this hypothesis (Juliano and Gravel 2002; Freeman and Byers 2006; Siesa et al. 2014), others did not (Antoń and Sniegula 2021; Amer et al. 2024). Hence, the importance of studying to what degree and in what direction IA predators differ from native predators in shaping local prey populations.

Predators can affect prey through direct consumptive effects or indirect non-consumptive effects (NCE). Predators can impose NCE by visual and/or chemical cues (kairomones), with the latter being unintentionally synthesised and released to the environment (Brown et al. 1970). By definition, kairomones evoke adaptive responses in prey (the receiver), increasing their chances of survival, while the release of these cues is maladaptive for the predator (Ruther et al. 2002). NCE might reduce prey population sizes to the same degree or, in some cases, to a higher degree than consumptive effects (Preisser et al. 2005; Preisser and Bolnick 2008; McCauley et al. 2011; Cinel et al. 2020; Sheriff et al. 2020). The NCE reduce prey fitness through risk-induced trait responses which affect prey fitness components, including behaviour, life history and physiology (Janssens and Stoks 2013; Garcia et al. 2017; Sniegula et al. 2019; Cinel et al. 2020; Yli-Renko et al. 2022; Wos et al. 2024). Although kairomones are often considered non-species-specific (Von Elert and Pohnert 2000), prey can respond differently to cues from various predator species, suggesting that the identity and composition of predator-released cues may vary (Turner et al. 1999; Van Buskirk 2001; Amer et al. 2024). Such variation may explain why prey exhibit different responses to alien predators that are chemically distinct from native species (Anton et al. 2020).

Kairomones are typically considered the primary drivers of NCE; however, predators can also host epibionts or promote microbial growth that may indirectly affect prey condition and survival, especially in aquatic systems (Ringelberg and Van Gool 1998; Dražina et al. 2018; Kumar et al. 2022). Such interactions highlight the multifaceted nature of predator-prey dynamics, suggesting that the effects observed may arise from a combination of chemical cues release by predators and biological contamination from epibionts.

While predator-induced changes in life history traits are well documented, effects of predators on physiological traits are less studied, yet also widespread (Hawlena and Schmitz 2010). While these may align and even explain effects on life history, they may also be decoupled (Slos et al. 2009; Raczynski et al. 2022) and indicate independent fitness-related effects that may become obvious only in the presence of other stressors. For example, NCE may reduce the prey energy content, thereby reducing their ability to deal with food stress and impair their immune and defence functions, thereby reducing the ability to deal with parasites, pathogens and toxic compounds (Stoks et al. 2006; Adamo 2022; Antoń et al. 2022).

NCEs might differentially affect prey traits during egg, larval and adult stages in prey with a complex life cycle (Sniegula et al. 2020; Amer et al. 2024; Supekar and Gramapurohit 2024). One reason is that different life stages typically differ in their risk of being preyed upon. Moreover, prey traits affected by NCE via exposure in a particular life stage can be coupled or decoupled with the following life stage. The coupling effect is assumed to be a result of latent or carry-over effects that link environmental stress in one life stage and phenotypic responses exhibited in later

stages (Stoks and Córdoba-Aguilar 2012; Moore and Martin 2020). Nevertheless, also decoupling across life stages has been hypothesised (Moran 1994). There is mixed empirical evidence supporting these alternative hypotheses. For example, negative effects of predation risk experienced during the larval stage carried over to negatively affect adult mass and energy storage in a damselfly (Stoks et al. 2006), but no such carry-over effect of predation risk experienced in the egg stage affected subsequent survival until emergence in mosquitoes (Fontana-Bria et al. 2017).

In the studies on carry-over effects in general and especially for those related to predation risk, the egg stage has been understudied and typically only a limited number of egg traits have been considered. This may result in unmeasured or hidden carry-over effects of exposure to predation risk from the egg stage. The egg stage is a key window where the consequences of individual experiences can have lifelong effects on behaviour, physiology and fitness (Kingsolver et al. 2011), as shown in several studies (Chivers et al. 2001; Sniegula et al. 2017, 2019). Exposure to predation risk may directly affect the egg stage by, for example, shortening or prolonging the egg development time (Blaustein 1997; Anderson and Brown 2009; Fontana-Bria et al. 2017; Sniegula et al. 2019; Amer et al. 2024) or changing the synchrony of hatching (Bozelli et al. 2008). What is more, the propensity of egg exposure to predation risk to carry over to larval performance may vary across predator species (Sih and Moore 1993; Antoł and Sniegula 2021; Amer et al. 2024), ecology (Bucciarelli et al. 2019) and invasion history of IA predators at a local scale (Anton et al. 2020; Mathers et al. 2022).

Here, we compare the NCEs imposed by native, alien and IA opportunistic omnivorous crayfish species (Kozák et al. 2015) on key damselfly egg life history and larval physiological traits in central Europe. This is an interesting predator-prey study system to address this topic as there is ample natural history information on both the crayfish predators (Twardochleb et al. 2013; Pacioglu et al. 2020) and the damselfly prey (Córdoba-Aguilar et al. 2022) and there is a well-resolved phylogeny (Crandall and De Grave 2017) and invasion history of the crayfish predators at a regional and local scale (Grabowski and Jażdżewski 2005; Śmietana 2011a, 2011b; Kouba et al. 2014). Previous studies indicated that the aquatic stages of damselflies can react to both native and invasive crayfish species in their life history traits (Siesa et al. 2014; Antoł and Sniegula 2021; Antoł et al. 2022; Palomar et al. 2023; Amer et al. 2024) and these responses were evident even at the level of gene expression (Wos et al. 2024). Yet, it is not clear whether such responses carry over across developmental stages and are present at the physiological level, which would broaden our understanding of the potential impact of exposure to alien and IA crayfish on natural prey populations.

Here, we focus on two fitness-related types of physiological traits, investment in immune function and energy storage, which have both been shown to be sensitive to predation risk in damselfly larvae (e.g. Stoks et al. (2006) Van Dievel et al. (2016)). Based on the naïve prey hypothesis (Cox and Lima 2006), we predicted (1A) the strongest effects on damselfly egg traits, i.e. egg development time until hatching, hatching synchrony and survival under the NCE of native crayfish species, weaker NCE of alien and IA crayfish species that has already invaded damselfly sites for several decades and the weakest or no NCE of IA crayfish species that has not yet invaded the damselfly sites. We further test whether exposure to native, alien and IA crayfish-associated chemical cues (CACC) in the egg stage generates carry-over effects into the larval stage. We predicted that (1B) exposure of eggs to

CACC released by native crayfish species will have negative carry over effect on larval mass, energy storage (measured as fat content) and investment in immune function measured as phenoloxidase activity, whereas exposure of eggs to CACC released by alien and IA crayfish will have weaker or no effects on larval traits because of damselfly naivety. Alternatively, alien and IA crayfish will cause (2A) stronger effects on egg traits and (2B) carry over effects on larval traits due to the absence of evolutionary exposure of prey to these predators (Sih et al. 2010; Anton et al. 2020; Antoł and Sniegula 2021; Amer et al. 2024), hence the opposite of the naïve prey hypothesis. Finally, the equally valid alternative hypothesis states that (3) the intensity of responses to CACC in the egg stage and carry-over effects in the larval stage will be based solely on predator phylogeny with relation to native crayfish species. Specifically, we expect that the closer the predator species' relatedness, the more similar the damselfly's response will be, due to the similar chemical composition of CACC (Anton et al. 2020).

Methods

Background

Ischnura elegans is one of the most common native damselfly species in central Europe. It lives in a variety of freshwater habitats, including lentic and lotic waterbodies (Dijkstra and Schröter 2020). Adult females commonly deposit eggs into decaying aquatic plants that flow on the water surface. The juvenile aquatic stage shares habitats with several top predator species (Corbet 1999), including fish and crayfish (Schaffner and Anholt 1998; Le Gall et al. 2017; Sniegula et al. 2019; Palomar et al. 2023). Both egg and larval stages react to these predator cues (Antoł and Sniegula 2021; Wos et al. 2023; Amer et al. 2024; Sniegula et al. 2024). Geographic dispersal and high gene flow, particularly at the local scale, have been documented in *I. elegans* (Babik et al. 2023) and this factor might contribute to the damselfly response to alternative predator species and types (i.e. native vs. IA).

We studied two crayfish species that are native to Europe: the noble crayfish (*Astacus astacus*) and the danube crayfish (*Pontastacus leptodactylus*). The noble crayfish species is listed as vulnerable in Europe on the IUCN Red List (Gherardi and Souty-Grosset 2010). It is protected by law in Poland, though its population numbers decline (Krzywosz and Śmietana 2004; Bonk et al. 2014; Stanek et al. 2015; Rozporządzenie Ministra Środowiska 2016). The danube crayfish originates from the Caspian Sea region and was introduced to central Europe in 19th century. Although the species is considered native to Europe, it is classified as alien in Poland. This is explained by the fact that the danube crayfish is alien for the Wisła and the Odra river drainages, covering the majority of Poland (Grabowski and Jażdżewski 2005; Kouba et al. 2014). Danube crayfish is one of the rarest and irregularly spread crayfish in Poland, which supports its non-invasiveness. The species is listed as least concern on the IUCN Red List (Gherardi and Souty-Grosset 2010) and is protected by law in Poland (Rozporządzenie Ministra Środowiska 2016). Both noble and danube crayfish species occupy ponds and rivers close to the damselfly sampling sites (Bonk M, unpublished data; Strużyński 2007) (Fig. 1).

The two studied IA crayfish species were the spinycheek crayfish (*Faxonius limosus*) and the signal crayfish (*Pacifastacus leniusculus*), which are both native to North America. The spinycheek species has been introduced to central Europe at

Phylogenetic relationship	Status	Present locally
Noble crayfish	Native	Yes
Danube crayfish	Alien	Yes
Signal crayfish	IA	No
Spinycheek crayfish	IA	Yes

Figure 1. Phylogenetic relationships amongst the studied crayfish species (modified from Crandall and De Grave 2017), their invasive status and presence at the local scale in Poland.

the end of 19th century and is currently the most common crayfish in EU countries, including Poland and the study region specifically (Śmietana 2011a; Kouba et al. 2014; World of Crayfish™ 2024). It occupies ponds and rivers close to the damselfly sampling sites (Fig. 1). It is noted as one of the most ferocious invasive invertebrates that causes decline of native species, including both prey and native crayfish populations (Nentwig et al. 2018) (Fig. 1). The signal crayfish has been introduced to northern Europe (Scandinavia) in the 1960s and, since then, it has spread across most of European countries (Śmietana 2011b). In Poland, the crayfish is found mainly in northern regions, but is expected to invade southern Poland, i.e. the damselfly sampling sites, in the near future; in 2020, new sites were found ca. 200 km west from where the damselflies have been collected (Barowska et al. 2023) (Fig. 1).

Phylogenetically, noble and danube crayfish are sister taxa and signal crayfish is from the same clade. In contrast, the spinycheek represents a different family and at the phylogenetic level is equally distanced from noble/danube and signal crayfish (Crandall and De Grave 2017) (Fig. 1).

Animal collection and rearing

Copulating adult female *Ischnura elegans* were collected using a butterfly net on 15 June 2021 from two nearby ponds in the city of Krakow, Poland: Mydlniki ponds (50°05'09.6"N, 19°50'21.8"E) and Bonarka pond (50°01'25.4"N, 19°57'06.5"E). We selected these ponds because they supported numerous *I. elegans* populations. Additionally, the availability of historical and current crayfish distribution data allowed us to explore possible effects of pond-specific history of crayfish and population differences in damselfly responses to chemical cues. Mydlniki ponds are sourced by the Rudawa River that holds native noble crayfish (A. Klaczak 2023, pers. comm). To our knowledge, no crayfish have been recorded in Bonarka pond. However, in a nearby pond (approx. 350 m away), the danube crayfish was recorded until 2019 (M. Bonk 2019, unpublished). This absence of crayfish in Bonarka pond suggests that damselflies from this site may not have co-evolved with crayfish predators, potentially resulting in increased stress responses to both native, alien and IA species. The uncertainty regarding crayfish presence in Mydlniki ponds might also contribute to naïve responses. On the other hand, strong gene flow between *I. elegans* populations, as shown in recent studies (Babik et al. 2023), could homogenise damselfly responses and limit local adaptations to specific predator types.

Field-collected female damselflies were put in plastic jars with moisturised filter paper for egg laying and transported by car to the laboratory at the Institute of Nature Conservation PAS in Krakow. Jars with females were placed in a room with a temperature of 22 °C and natural day light. In total, 19 females from Mydlniki pond and 12 females from Bonarka pond laid large (> 100 eggs/clutch) egg clutches between 16 and 17 June 2021. These clutches were used in the experiment.

All crayfish species were collected in the field and transported by car to the laboratory several weeks prior the start of the experiment. Noble and danube crayfish were collected from a private pond near the town Miejska Górka (51°39'13.2"N, 16°58'52.3"E), spinycheek crayfish were collected from an excavation pond in Kryspinów (50°02'56.8"N, 19°47'28.7"E) and signal crayfish were collected from Hańcza Lake (54°15'31.9"N, 22°48'51.9"E). Noble, spinycheek and signal crayfish were collected and housed with permissions from, respectively, General Directorate of Environmental Protection in Warsaw (per. DZP-WG.6401.147.2021.TŁ), Regional Directorate of Environmental Protection in Krakow (per. OP.672.4.2021.GZ) and Regional Directorate of Environmental Protection in Białystok and Krakow (per. WPN.6205.21.2020.ML and OP-I.672.8.2020.MK1).

The densities of crayfish in aquaria were based on the basal metabolic rate equations obtained for crayfish (Wheatly 1989). After weighing, we kept two specimens of noble, danube and signal crayfish (wet mass ca. 100 g for each species) and five specimens of spinycheek crayfish (wet mass ca. 100 g) per experimental aquarium. Crayfish were fed with fish food pellets twice per week and live chironomid larvae once per week.

At egg laying, every clutch (= family) was divided into five treatment groups, with 20 eggs per family per treatment. At hatching, these five egg-treatment groups were further split into two larval-treatment subgroups: a control group or a crayfish-exposure group. In the control subgroup, larvae were not exposed to CACC, allowing us to test for carry-over effects of predator exposure during the egg stage. Larvae in the CACC subgroup received the same crayfish treatment as in the egg stage. This resulted in nine treatment groups: control(egg) – control(larva), noble(egg) – control(larva), noble(egg) – noble(larva), danube(egg) – control(larva), danube(egg) – danube(larva), spinycheek(egg) – control(larva), spinycheek(egg) – spinycheek(larva), signal(egg) – control(larva) and signal(egg) – signal(larva) group (Fig. 2). Throughout the experiment individuals were followed at the family level. Eggs were moved to separate 200 ml drinking cups (height – 9 cm, depth – 4 cm) creating sets of 20 eggs/cup. Accidentally, 15 out of 142 cups contained more than 20 eggs, which was accounted for in the statistical analysis by including egg density as a covariate in our models. Every cup was filled with 67 ml of dechlorinated tap water and 33 ml of treatment water with or without CACCs. To introduce the CACCs, we used water from the aquaria holding crayfish. As a control, we used dechlorinated tap water held in the same type of aquarium as the aquaria with crayfish. We placed cups with eggs in an incubator (ST700, Pol-Eko) at a constant temperature of 20 °C and a photoperiod of L:D 16:8 h. The cups were randomly distributed to the treatments. We replaced 33 ml of water in cups with water from the appropriate crayfish species and control aquarium every second day. The median half-life of predator cues is ca. 48 h (Van Buskirk et al. 2014). The effectiveness of the here-applied CACC refill frequency has been confirmed in previous experiments on damselfly eggs and larvae (Sniegula et al. 2019; Raczynski et al. 2022; Amer et al. 2024).

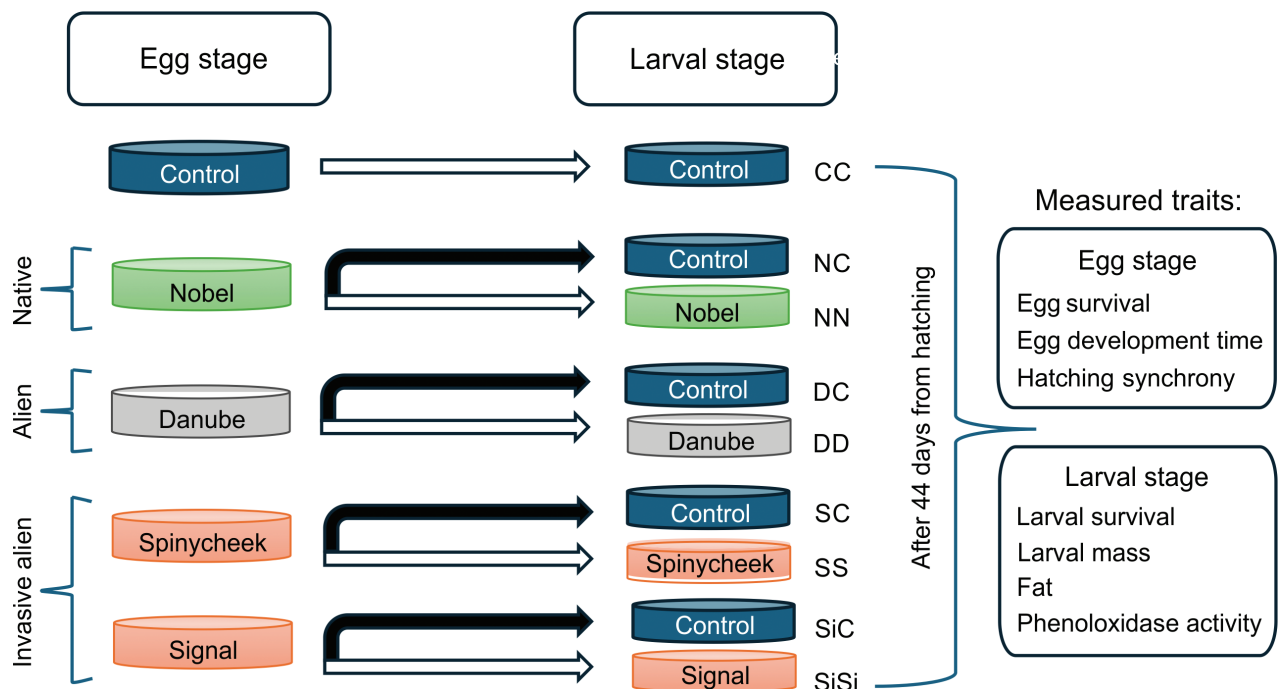


Figure 2. A scheme of the experimental method, showing egg and larval crayfish treatments and the traits measured 44 days after hatching. Filled arrows indicate carry-over non-consumptive effect (NCE), empty arrows indicate continuous exposition to NCE. Abbreviations for the crayfish treatment groups are indicated to the right of the larval treatment groups: CC – control(egg)-control(larva), NC – noble(egg)-control(larva), NN – noble(egg)-noble(larva), DC – danube(egg)-control(larva), DD – danube(egg)-danube(larva), SC – spinycheek(egg)-control(larva), SS – spinycheek(egg)-spinycheek(larva), SiC – signal(egg)-control(larva) and SiSi – signal(egg)-signal(larva) group.

The number of larvae hatched per cup ranged from 2 to 34. The larvae were fed *ad libitum* daily with laboratory-cultured *Artemia* nauplii. When the earliest hatched larvae in each cup reached the age of 44 days, all larvae from the same cup were group-weighted and frozen in the same Eppendorf tube at -80 °C for physiological analyses. We chose this larval age for two reasons: it represented approximately 50% of the larval development time until emergence and each group had reached the minimal wet mass threshold for the analysis of physiological traits.

Response variables

The proportion of eggs that survived per cup was calculated as the number of eggs per cup that hatched. The unhatched eggs were considered as dead. We noted the egg development time from egg laying to hatching. Every cup was checked for new hatchlings every morning and afternoon, with half a day used as the measurement unit. Based on the egg development times in a given cup, we estimated hatching synchrony per cup as the coefficient of variation (CV); the smaller the CV, the higher the hatching synchrony. This trait is relevant to measure because it can represent one of the preys' tactics for escaping predation pressure, for example, predator satiation effect (Janzen 1971) or bet hedging tactic (Simons 2011). Larval survival was measured as the number of larvae per cup that survived until day 44 after the first individual in the cup hatched. Mean larval wet mass per cup was measured when the first larva in that cup reached the age of 44 days after hatching and was calculated as the total mass divided by the number of larvae per cup (1–8 larvae per cup).

Physiological traits

We assessed physiological traits from the body supernatants of preserved larvae. To prepare the body supernatant, the larvae were homogenised in PBS buffer (Phosphate-Buffered Saline, final mass \times 15 μ l PBS) and subsequently centrifuged.

As a measure of investment in immune function, we quantified the activity of phenoloxidase (PO). This enzyme plays a key role in the defence of insects against bacterial, fungal and viral agents (González-Santoyo and Córdoba-Aguilar 2012). The PO activity assay followed the method described by Stoks et al. (2006). In this assay, 10 μ l of the homogenate was combined with 105 μ l of phosphate-buffered saline (PBS) and 5 μ l of chymotrypsin and the mixture was incubated for 5 minutes in a 384-well microtiter plate. Subsequently, L-DOPA (1.966 mg dihydroxyphenyl-L-alanine per 1 ml of PBS buffer) was added to the samples. The linear increase in absorbance at 490 nm was measured every 20 seconds for 30 minutes at 30 °C. The average of the duplicate readings for each sample was used for statistical analyses. PO activity was expressed in nmol of dopachrome formed per minute. To normalise PO activity, the protein content in the supernatant of each sample was measured using the Bradford method (Bradford 1976).

We determined the fat content of damselfly larvae using a modified protocol based on Marsh and Weinstein (Marsh and Weinstein 1966), as described by Verheyen et al. (Verheyen et al. 2018). Small glass tubes were filled with 8 μ l of supernatant and 56 μ l of concentrated sulphuric acid (100%). The tubes were heated at 150 °C for 20 minutes, then allowed to cool before adding 64 μ l of milliQ water. A 380-well microtiter plate was loaded with 30 μ l of the final mixture per larva in triplicate and absorbance was measured at 490 nm. The mean of the three readings was used for statistical analyses.

Statistical analyses

All the tests were performed using R version 4.3.2. Following packages were used: the lme4 package for general linear mixed models (Bates et al. 2015), the car package for estimating p-values (Fox and Weisberg 2019) and the *summary* function for checking contrasts between different levels (specifically, between control and different CACC treatments and between ponds). For the graphics, the ggplot2 package was used (Wickham 2016). We assessed the homogeneity of variance and the normality of residuals by visually examining the residual plots. In a separate analysis for egg and larval survival, proportions of surviving eggs or larvae per cup were response variables (both arcsin transformed) and CACC treatment (five levels for the egg stage and nine levels for the larval stage) and pond (two levels) were explanatory variables. Similar tests, but with no transformation of response variables were used for analysing the hatching synchrony, egg development time, mean larval mass per cup, mean fat storage per cup and mean PO activity per cup. As, at the end of the experiment, cups held different number of larvae, analyses of larval mass, total fat content and PO activity per cup were corrected by the number of larvae per cup. In all models, family nested in pond was added as random effect. We initially fitted global models that incorporated all main effects and interaction terms. Interaction terms with p-values greater than 0.05 were then excluded from the final models.

Results

The number of individuals considered in the analyses ranged from 86 to 339 (egg treatments) and from 5 to 10 (cumulative egg and larval treatments) per treatment combination. Suppl. material 1: table S1 shows the number of individuals across all treatment groups.

The analyses showed significant main effects of CACC on egg and larval life history and physiological traits, as well as interacting effect of CACCs and pond on egg life history in *I. elegans*.

Family (random effect) explained 21.5% of the variance in egg development time and 17.7% in larval mass. Variance explained by family was lower for other traits and models for hatching synchrony and larval fat content indicated negligible family-level variance (model singularity). The variance explained by family was generally higher after accounting for fixed effects, such as CACC treatment and pond, which indicated the role of experimental treatments in shaping observed variability. A table summarising the variance explained by family is available in Suppl. material 1: table S2.

Effects during the egg stage

In general, CACC had a negative effect on egg survival (Fig. 3A, Table 1). This significant result was mainly caused by the signal CACC, which decreased egg survival by half and the danube CACC, which decreased egg survival by a fourth compared to the control group. Noble and spinycheek CACC did not affect egg survival (Fig. 3A, Suppl. material 1: table S3). The two pond populations did not differ in egg survival (Fig. 3A, Table 1).

Overall, eggs took longer to develop under the CACC treatment. This result was especially pronounced under the signal CACC (+10 days), which caused the longest egg development time, followed by the danube (+7 days), spinycheek (+4 days) and noble (+2 days) CACC. These results were supported by Tukey's HSD pairwise comparisons (Fig. 3B, Suppl. material 1: table S4). The significant interaction between CACC and pond indicated that the effect of signal CACC cue (compared to the pond control) is stronger in Mydlniki pond than in Bonarka pond. Yet, the ponds did not differ from each other for a given CACC treatment (Suppl. material 1: fig. S1, table S4; Table 1).

Hatching was about two times more synchronised under the control treatment than in the presence of CACC (Fig. 3C, Table 1). The hatching synchrony did not differ between any of the treatments with CACCs (Suppl. material 1: table S5). Ponds did not differ in hatching synchrony (Fig. 3C, Table 1).

Effects during the larval stage

In general, exposure to CACCs decreased larval survival when quantified when the first larva in a cup reached an age of 44 days (Fig. 4A, Table 2). The decreased larval survival only occurred in response to danube and signal CACC and this both under combined egg-larval exposure (danube-danube and signal-signal CACC treatments) and under exposure of only the eggs (danube-control and signal-control CACC treatments), the latter indicating carry-over effects. In contrast, survival was not affected by exposure to noble and spinycheek CACC (noble-control, noble-noble, spinycheek-control and spinycheek-spinycheek CACCs) (Fig. 4A, Suppl. material 1: table S6). Damselfly larvae of both ponds did not differ in survival across all treatments (Table 2).

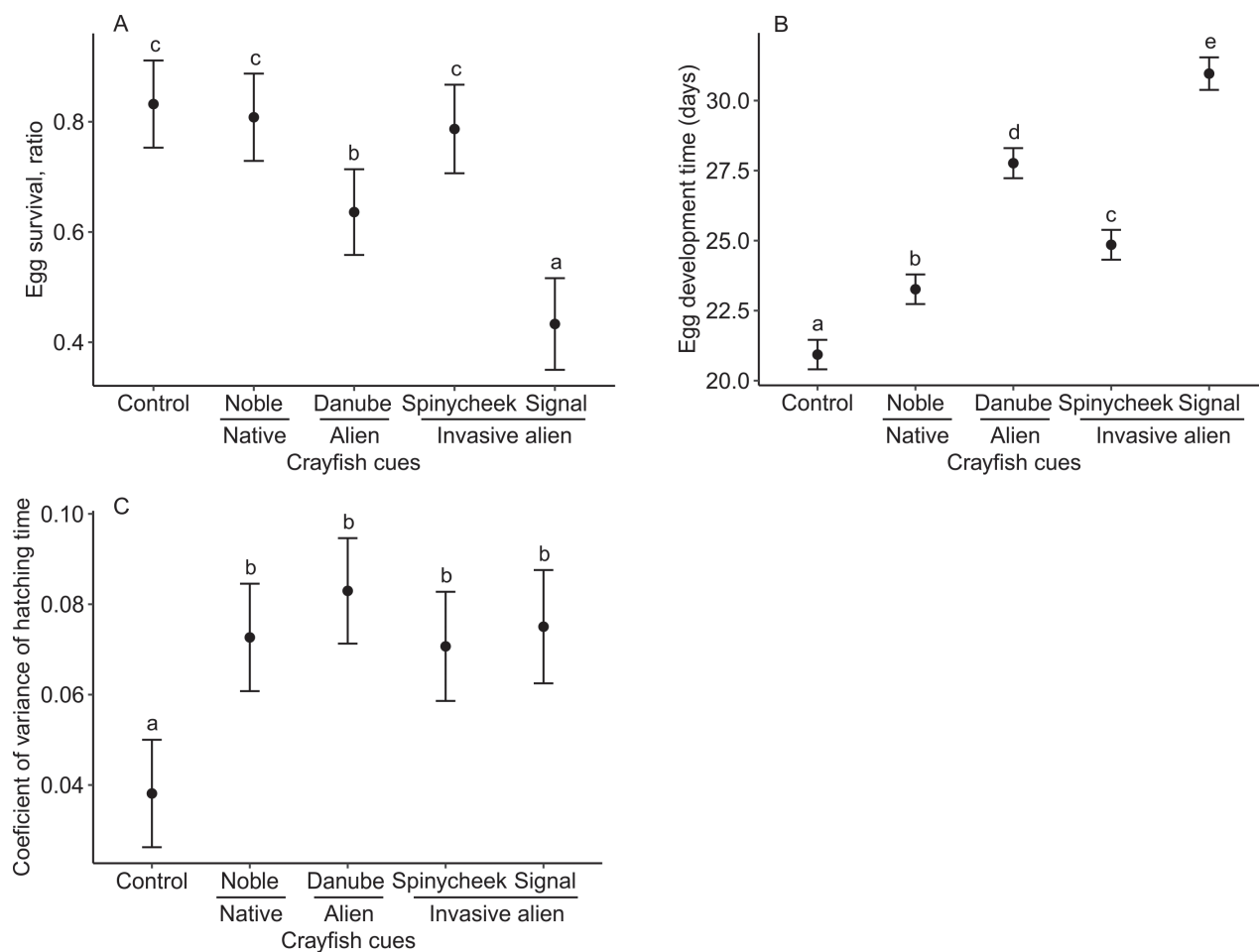


Figure 3. Effects of crayfish cues from native and invasive alien (IA) crayfish species on the egg survival rate (A), development time (B) and hatching synchrony (C) in *I. elegans*. Shown are means with 95% CI. Different letters indicate means that are significantly different, based on Tukey pairwise tests.

Table 1. Effects of crayfish-associated chemical cues (CACCs), pond and their interactions on egg survival, development time in days and hatching synchrony measured as coefficient of variation (CV) in *I. elegans*. Significant p-values are in bold.

Predictor	df	Chisq	p-value
Egg survival			
CACCs	4	78.8	< 0.001
Pond	1	0.2	0.680
Egg development time			
CACCs	4	1206.8	< 0.001
Pond	1	0.9	0.327
CACCs × pond	4	10.8	0.028
Hatching synchrony, CV			
CACCs	4	33.6	< 0.001
Pond	1	0.1	0.814

CACC decreased larval mass (Fig. 4B, Table 2). This mass decrease was especially pronounced under combined egg and larval exposure in the noble-noble, danube-danube and signal-signal CACC treatments and less so, but still significantly, under only egg exposure in the danube-control CACC treatment, indicating a carry-over effect. Exposure to spinycheek CACC never affected larval mass

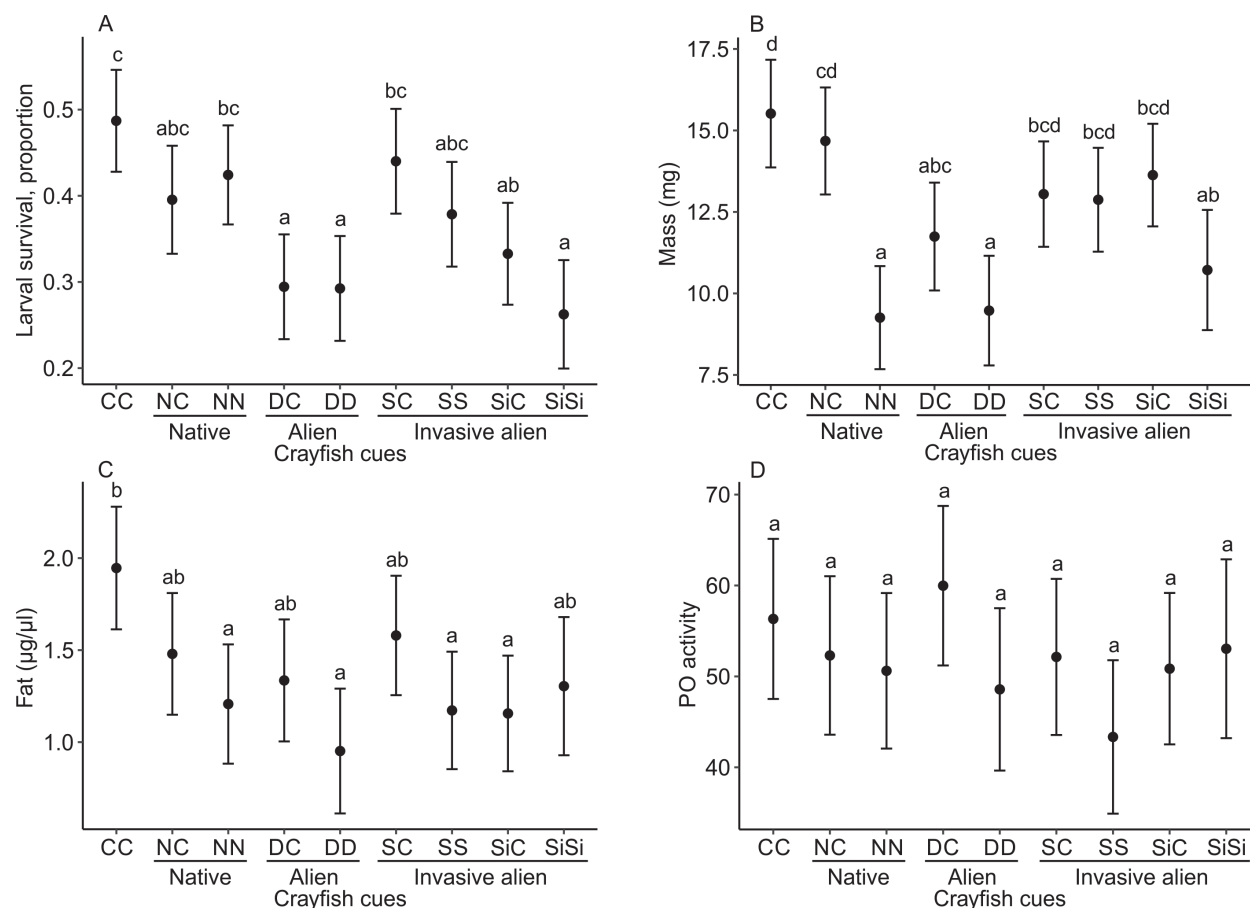


Figure 4. Effects of crayfish-associated chemical cues (CACCs) from native and invasive alien (IA) crayfish on the larval survival rate (A), mass (B), fat content (C) and phenoloxidase activity (D) in *I. elegans*. Note that in treatment combinations where the second letter is “C” (hence NC, DC, SC and SiC), the larvae were only exposed to the CACCs in the egg stage, but not in the larval stage, hence, when different from the control CC treatment would indicate a carry-over effect from egg exposure. Shown are means with 95% CI. Different letters indicate means that are significantly different, based on Tukey pairwise tests. Abbreviations for CACCs along the x-axis are as in Fig. 2.

Table 2. Effects of crayfish-associated chemical cues and pond on larval survival and mean values of mass, fat content and phenoloxidase activity (PO) per cup. Analyses on larval mass, fat content and PO were corrected by number of larvae per cup. Significant p-values are in bold.

Predictor	df	Chisq	p-value
Survival			
CACCs	8	56.7	< 0.001
Pond	1	0.2	0.675
Mass			
CACCs	8	67.6	< 0.001
Pond	1	0.1	0.732
No. of larvae per cup	1	175.6	< 0.001
Fat content			
CACCs	8	22.3	0.004
Pond	1	1.1	0.299
No. of larvae per cup	1	2.7	0.102
Phenoloxidase activity			
CACCs	8	10.9	0.211
Pond	1	0.3	0.592
No. of larvae per cup	1	31.4	< 0.001

(spinycheek-spinycheek, spinycheek-control) (Fig. 4B, Suppl. material 1: table S7). Damselfly larvae of both ponds did not differ in mass across all treatments (Table 2).

CACC negatively affected the total fat content (Fig. 4C, Table 2). The fat content decrease was especially pronounced under combined egg and larval exposure in the noble-noble, danube-danube and spinycheek-spinycheek CACC treatment, with the exception of the signal-signal CACC treatment where the effect was absent. Under only egg exposure treatment, the only significant effect was found under signal-control CACC treatment, indicating a carry-over effect (Suppl. material 1: table S8). Damselfly larvae of both ponds did not differ in fat content across all treatment groups (Table 2).

CACC did not affect phenoloxidase activity (PO) (Fig. 4D, Table 2), which was also supported in a Tukey's HSD pairwise comparisons (Suppl. material 1: table S9). Damselfly larvae of both ponds did not differ in PO across all treatments (Fig. 4D, Table 2).

Discussion

We examined the non-consumptive effects (NCEs) of native, alien and invasive alien (IA) crayfish species on the egg and larval traits of the damselfly *I. elegans*, with special attention for potential carry-over effects from the egg to the larval stage. Our results do not support the first (prey naivety) hypothesis stating that alien and IA crayfish species exert weaker effects on damselfly egg survival and development time than native crayfish species. Instead, the strongest effects were observed in response to the IA signal crayfish-associated chemical cues (CACCs), which has not yet invaded the damselfly sampling ponds, supporting one of our alternative hypotheses. Eggs exposed to these cues exhibited the longest development time, lowest survival and disrupted hatching synchrony. While we did detect negative carry-over effects on larval survival and mass in response to egg exposure to CACCs, this was only the case for cues from one alien crayfish (danube) and one IA crayfish (signal), supporting the alternative hypothesis (i.e. the opposite of the prey naivety hypothesis). Interestingly, only egg exposure to IA signal CACCs induced a negative carry-over effect on larval fat content. These are important results for invasion biology as they suggest that crayfish-induced NCE cannot only persist across life stages, but are also not restricted to native predators. Finally, there was limited support for the last alternative hypothesis, as the observed responses did not strictly follow phylogenetic relationships. In other words, closely-related crayfish species did not consistently elicit similar prey responses across all traits assessed.

Immediate NCE effects on the egg traits

Our findings underscore the importance of studying egg-stage predator-prey interactions in species with complex life cycles, as exposure during the egg stage can significantly influence fitness-related traits. CACC from the IA signal crayfish reduced by half egg survival and extended the egg development time by 10 days, indicating that the mere presence of IA predator-associated chemical cues can induce strong stress responses in damselfly eggs. Such responses are consistent with other studies demonstrating that exposure to predator cues during the early life stages can trigger significant physiological changes that decrease egg survival (Blaustein 1997; Miner et al. 2010; Sniegula et al. 2019). However, the reduction

in egg survival does not align with the classical definition of kairomones, which indicates that prey responses should be adaptive (Ruther et al. 2002). In this case, the CACCs appear maladaptive for the prey, as the observed mortality does not confer any immediate survival benefit.

Interestingly, CACC from the alien danube crayfish, which has been present in the region for over a century (Strużyński 2007), also reduced egg survival and extended development time, though to a lesser degree. This happened despite the fact that the danube crayfish is known for being mild, relatively less active when feeding and has an R-reproductive strategy, which is in conflict with other alien crayfish species in Europe, including signal crayfish (Pacioglu et al. 2020; Galib et al. 2022). However, similar to the signal crayfish, the danube crayfish is one of the rarest crayfish species in Poland, with a rather irregular distribution. This may lead to a low overlap between local populations of the damselfly and the crayfish and, consequently, the damselfly eggs' response to this alien species may be similar to their response to IA species.

In contrast, native noble crayfish and locally invading IA spinycheek crayfish had no effect on egg survival. This suggests that the eggs from the studied damselfly populations may have evolved some resistance to the NCEs of these crayfish or that these species produce weaker CACCs that do not cause strong antipredator egg responses (Anton et al. 2020). Additionally, the ecological relevance of these predators may play a role: signal crayfish, which can strongly alter aquatic ecosystems (Nyström et al. 1996; Galib et al. 2022), may represent a higher threat to damselfly eggs than either the native noble crayfish or IA spinycheek crayfish, leading to a stronger innate response (Lavery et al. 2015).

The eggs of *I. elegans* prolonged development times under exposure to CACCs and this across all treatment groups, yet, with significant differences between native, alien and IA crayfish. Eggs exposed to IA signal CACCs showed the longest delay, whereas native noble CACCs caused the shortest delay, but still significant. This variation suggests that damselfly eggs exhibit flexible plasticity in response to predation risk and that the imposed risk is the highest under IA crayfish (Cox and Lima 2006; Sih et al. 2010). A strong delay of egg development time under signal CACCs was earlier shown in other populations of *I. elegans* (Antoń and Sniegula 2021; Amer et al. 2024), confirming that predator-induced stress responses are consistent across populations and may represent an adaptive mechanism to cope with novel predation stress. However, the opposite pattern with shorter *I. elegans* egg development under spinycheek CACCs was previously reported (Antoń and Sniegula 2021), indicating population specific responses likely associated with habitat-specific predator history (Anton et al. 2020; Mathers et al. 2022). It might be argued that prolonged egg development in the presence of cues from egg predators may carry costs. Extended egg development would indeed increase exposition time to crayfish predation (Sih and Moore 1993). This may explain why green frog (*Rana clamitans*) and East African reed frog (*Hyperolius spinigularis*) eggs hatched earlier when exposed to egg predators (Vonesh 2005; Anderson and Brown 2009). However, prolonged development times under egg predation risk may serve as a defence strategy to reduce the likelihood of hatching into high-risk larval environments (Ferrari et al. 2010). Furthermore, delayed hatching may occur as a non-adaptive result of stress-induced re-allocation of energy to costly defence mechanisms against predators (Hawlena and Schmitz 2010), away from investing in a fast embryonic development rate. It might also be that cues from

generalist predators like crayfish, that are capable of preying on both egg and larval stages of the damselfly, mediate the egg response (discussed below).

The observed disruption in hatching synchrony under CACC exposure, with similar strength across all treatment groups, indicates that predator-associated cues may also affect egg cohort timing. Reduced hatching synchrony can have ecological implications, as it may reduce the effectiveness of antipredator strategies like predator satiation (Simons 2011). Studies on other *I. elegans* populations indicated that the damselfly hatching synchrony under predation stress from IA signal crayfish and native perch cues did not deviate from the control treatment (Sniegula et al. 2019; Antoł and Sniegula 2021). The discrepancy between previous and current results suggests a population specific response to predator-associated cues which might be linked to the predator history at a specific site (Anton et al. 2020).

Carry-over and cumulative NCEs on larval traits

Our study showed significant carry-over effects from the egg stage to the larval stage when *I. elegans* eggs were exposed to CACCs from native, alien and IA crayfish species. Larvae that were only exposed to CACCs during the egg stage showed reduced survival, lower body mass and reduced fat content compared to control groups, indicating that predator-induced stress effects can persist across life stages. Notably, the strength of these carry-over effects varied amongst the three crayfish types, with the most pronounced negative effects observed for the alien danube and IA signal CACCs. This pattern aligns with previous research suggesting that alien and IA predators may elicit stronger stress responses due to the absence of evolutionary exposure of prey to these predators (Cox and Lima 2006; Sih et al. 2010; Antoł and Sniegula 2021; Amer et al. 2024; Sniegula et al. 2024). The carry-over effects are consistent with the idea that stress experienced during the egg stage, including rarely documented for egg stress imposed by predation risk, can persist and manifest in later stages (Stoks and Córdoba-Aguilar 2012; Moore and Martin 2020; Sniegula et al. 2020).

Our study also indicated that larvae exposed to CACCs during both the egg and larval stages exhibited greater reductions in mass and fat content than those only exposed in the egg stage. This cumulative effect of exposure to predator-associated chemical cues indicates that the stress induced during the egg stage was not softened after hatching and that continuous exposure further intensifies the negative effects. For instance, larvae that experienced noble, danube and signal CACCs during both stages showed significantly lower mass across all treatment groups. The negative effect of predator stress on prey mass or size was earlier shown in other damselflies species and semi-aquatic insects such as mayflies (McPeck et al. 2001; Peckarsky et al. 2002). Our results add to the knowledge that continuous exposure to predator-associated cues during both egg and larval stages intensifies the physiological cost of antipredator defences.

The significant reduction in larval fat content in response to CACCs as observed in our study provides further evidence that predator-induced stress can disrupt energy allocation across life stages. In semi-aquatic invertebrates, fat reserves are critical for sustaining growth and immune function during the larval stage (Stoks et al. 2006) and their depletion due to continuous stress can impair development. In particular, the larvae exposed during both egg and larval stages to the native noble, alien danube and IA spinycheek CACCs exhibited the greatest reduction in fat content, suggesting that alien and IA crayfish impose similar physiological costs

compared to native crayfish. The fact that continuous exposure to alien and IA CACCs resulted in the strongest carry-over effects suggests that invasive predators may have long-term consequences for prey populations. Further research should explore whether these carry-over effects also bridge metamorphosis and translate to reduced reproductive success in the adult stage, which could have implications for population dynamics in ecosystems invaded by alien IA crayfish.

Finally, we found no significant effect of predator-associated cues on phenolox-
idase activity, our measure of investment in immune function. This happened
probably because there was apparently no effect of continuous exposure and so no
immediate effect, which may explain also the absence of any delayed effects. This
absence of an effect on immune parameters may reflect the complex and variable
nature of carry-over effects, where some traits, such as energy storage and total
body mass, are more susceptible to early-life stressors than others.

Conclusions

Our study underscores the impacts of predator exposure in species with complex life cycles, where early-stage stressors can persist and negatively affect later stages. These findings are particularly relevant for predicting how non-native species, such as the IA crayfish, can alter prey populations through non-consumptive effects that accumulate over time. Given that alien and IA predators imposed stronger selective pressures on naïve prey, as found in terms of a higher effect on egg and larval survival, egg development time and larval mass and fat content, it is crucial to incorporate these carry-over and cumulative stress effects into ecological models to better predict population responses and ecosystem dynamics under biological invasions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: SS. Data curation: AA, SS. Formal analysis: SS. Funding acquisition: SS. Investigation: SS, AA, NRA, DK. Methodology: SS, RS, AA. Project administration: SS. Resources: SS. Supervision: SS. Visualization: NRA (Figure 2), SS. Writing – original draft: SS. Writing – review and editing: RS, DK, MB, NRA, AA, SS.


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Data availability

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

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Supplementary material 1

Supplementary data

Authors: Szymon Sniegula, Dorota Konczarek, Maciej Bonk, Andrzej Antoł, Nermeen R. Amer, Robby Stoks

Data type: docx

Explanation note: An additional figure showing the effects of crayfish cues and the pond of origin on egg development time in *I. elegans*, as well as tables showing the variance explained by family (random effect) for different response traits, number of individuals per treatment group and collection pond, and multiple comparisons of proportions using Tukey's HSD contrasts.

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Supplementary material 2

Raw data used for the statistical analysis

Authors: Szymon Sniegula, Dorota Konczarek, Maciej Bonk, Andrzej Antoł, Nermeen R. Amer, Robby Stoks







Data type: xlsx

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Research Article

Habitat and diet interactions in a lowland temperate river suggests no direct impact of non-native monkey goby (*Neogobius fluviatilis*) on native spined loach (*Cobitis taenia*)

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Abstract

Non-native species can negatively impact native ecosystems, but their introduction does not always lead to major ecological changes. This study examines interactions between the non-native monkey goby (*Neogobius fluviatilis*) and the native spined loach (*Cobitis taenia*) through field observations and controlled laboratory experiments. We assessed microhabitat use, dietary similarities, and behavioral interactions between the two species. Field results revealed overlapping microhabitats dominated by coarse sand, but with no significant displacement of the spined loach by the monkey goby. Laboratory trials further supported these findings, showing no competitive interference in habitat selection, with both species co-occupying preferred substrates (coarse sand). Diet analysis indicated limited trophic overlap (17%), suggesting resource partitioning rather than direct competition. The results show no evidence that the non-native monkey goby exerts direct harmful effects on the native spined loach in the river studied. Instead, our study highlights the potential for coexistence, emphasizing the need for nuanced approaches in assessing the ecological impacts of non-native species. However, it should also be noted that our results are time and space-limited, and indirect and/or long-term effects, not captured by this study, may exist. Current research contributes to a broader understanding of complex biotic interactions between non-native and resident species.

Key words: Competition experiment, diet analysis, stable isotopes, substrate preferences



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Introduction

Increasing anthropogenic pressure significantly affects aquatic ecosystems, leading to homogenization through habitat modifications and species invasions (Marr et al. 2013). In response, extensive efforts have been made globally and nationally, including the implementation of regulations, risk assessments of non-native species, and management strategies aimed at eradicating or controlling their populations (Britton 2023). Despite these efforts and advancements in understanding the biology of non-native species, their proliferation persists, highlighting the urgent need for studies confirming their impact to inform and implement effective control measures.

The most successful invaders of European inland waters include Ponto-Caspian gobies, with the round goby *Neogobius melanostomus*, serving as a prominent model species due to its significant negative impacts (Cerwenka et al. 2023). Conversely, other goby species such as the monkey goby *Neogobius fluviatilis*, racer goby *Babka gymnotrachelus*, bighead goby *Neogobius kessleri*, and western tubenose goby *Proterorhinus semilunaris* have been relatively understudied despite actively expanding their ranges (Grabowska et al. 2023). In Europe, the primary pathways for the expansion of Ponto-Caspian gobies are the central and southern corridors (Bij de Vaate et al. 2002; Semenchenko et al. 2011). Over the past three decades, the monkey goby, racer goby, bighead goby, and western tubenose goby have utilized those corridors to disperse. While the round goby typically remains in main rivers, the other gobies successfully expanded into tributaries.

The ecological impact of the monkey goby remains poorly understood. Experimental laboratory studies have not shown any detrimental effects on native species, such as the European bullhead *Cottus gobio* (Błońska et al. 2016), river bullhead *Cottus perifretum* (Van Kessel et al. 2011), stone loach *Barbatula barbatula* (Van Kessel et al. 2011). Irrespective of the season (spawning / non-spawning) the monkey goby influence on the native counterpart was similar to that of conspecifics (Błońska et al. 2016). Other studies showed the advantages of the monkey goby over its native analogue species consisting of lower energy expenditures on metabolism under increased temperature (Kłosiński et al. 2024) and enhanced boldness and exploratory behavior, allowing it to expand across the open riverbed (Augustyniak et al. 2024).

Given that the monkey goby is constantly and successfully increasing its non-native range, establishing abundant populations (e.g. in Poland it covered 340 km in five years; Bylak and Kukuła 2024) it is unlikely to assume that its presence is indifferent to native species, in particular those of similar requirements. Apart from the already existing intraspecific competition, the arrival of non-native gobies can add to the interspecific competition, though it would vary with season and fish species (Błońska et al. 2016). All the above mentioned research on interactions between native fish species and the monkey goby was performed under controlled laboratory conditions (Van Kessel et al. 2011; Błońska et al. 2016; Kłosiński et al. 2024), necessitating a more comprehensive approach that includes both field and laboratory observations to present not only potential, but also direct influences of the invasive species. Such an approach reveals that instead of competition suggested based on lab experiments, an alternative scenario is quite often implemented in ecosystems after arrival of an invasive species, i.e. resource and habitat partitioning to avoid competition with ecologically similar native species (Tran et al. 2015; Kakareko et al. 2016; Britton et al. 2018). However, the impact of non-native species is known to be context- and site-dependent. The same species might pose a serious threat in one location while having a mild effect in another (Błońska et al. 2024).

One of the native fish species that can be potentially affected by the expansion of the monkey goby invasion is the spined loach *Cobitis taenia* (Błońska et al. 2024). Both species inhabit a wide range of freshwaters, including rivers, lakes and reservoirs (Robotham 1978; Grabowska et al. 2023). They have similar habitat preferences, e.g. for sandy bottom (Robotham 1978; Copp and Vilizzi 2004; Pietraszewski 2015; Płachocki et al. 2020) and a habit of burying themselves in the substrate, possibly as an antipredator strategy (Kakareko 2011 in case of gobies). Besides, both feed on benthic macroinvertebrates, mainly chironomid larvae (Marszał et al. 2003; Grabowska et al. 2009; Jazdzewski 2020; Didenko et al. 2021). Faunistic fish studies

showed that the spined loach co-occurred with the monkey goby at each of 22 sites sampled along the distance of almost 360 km in the Eastern Bug River (Penczak et al. 2010). Their generally similar lifestyle and requirements suggest that they should have equally similar patterns of in-stream distribution and abundance that can potentially result in competitive interactions. The spined loach occurs across almost the whole of Europe and central Asia (Bohlen and Ráb 2001; Janko et al. 2007), but is endangered in many European countries (Kotusz 1996). The species is listed in Appendix III of the Bern Convention and Annex II of the EC Habitats Directive (92/43/EEC) on the Conservation of Natural Habitats and of Wild Fauna and Flora. The rapid spread of the monkey goby in the Pilica River has coincided with the spined loach decrease (own unpublished data). On the other hand, the observed decline of spined loach abundance and occurrence might be caused by the synergic effect of many other factors that alter environmental conditions in a river negatively for spined loach, and the arrival of the non-native species may only contribute to the decline or be not harmful at all. The causal relationship between these phenomena is unknown, as the interactions between these two species have never been investigated.

Therefore, we conducted an extensive study on the interactions between the monkey goby and spined loach to fill this knowledge gap and verify the impact of the monkey goby. Our study combines field observations of microhabitat occupation and diet overlap at sites of co-occurrence, utilizing traditional stomach content analysis and contemporary stable isotope analysis, along with laboratory experiments on habitat preferences and competition for limited resources. We hypothesised that (i) the monkey goby will competitively displace the native spined loach through interference competition and that (ii) the monkey goby and spined loach have overlapping dietary niches, indicating potential competition for food resources. We tested the first hypothesis in the laboratory through direct assessment of competition for limited habitat resources and in a field study, investigating the co-occurrence of both species in the same microhabitats. The second hypothesis was tested with stable isotope analysis and stomach content analysis.

Materials and methods

Field research

Field campaign to collect data on species occurrence was conducted in May 2024. May was chosen to collect samples to avoid peaks of macroinvertebrate density in early spring (before the emergence of diapaused generations) and in summer (after emergence and growing new generations) (Murphy and Giller 2000). Specimens of monkey goby and spined loach were sampled from the Pilica River near Spała village, Poland (Fig. 1), by electrofishing (EFGI 650; BSE Specialelektronik Bretschneider, Germany) using point sampling along the river stretch of 100 × 4 m (length and width, respectively; 95 points in total). Electrofishing, even with a low-power backpack electrofisher as used in our study, could potentially lead to localized avoidance behavior in fish, where individuals escape the immediate area of the anode and move to nearby unsampled locations. This may result in a slight underrepresentation of certain individuals at sampling points or influence their spatial distribution. However, we believe that the potential effects of this bias are mitigated for the following reasons: (1) wading upstream ensures that only fish in close proximity to the anode are affected; and (2) the specific behavior of focal

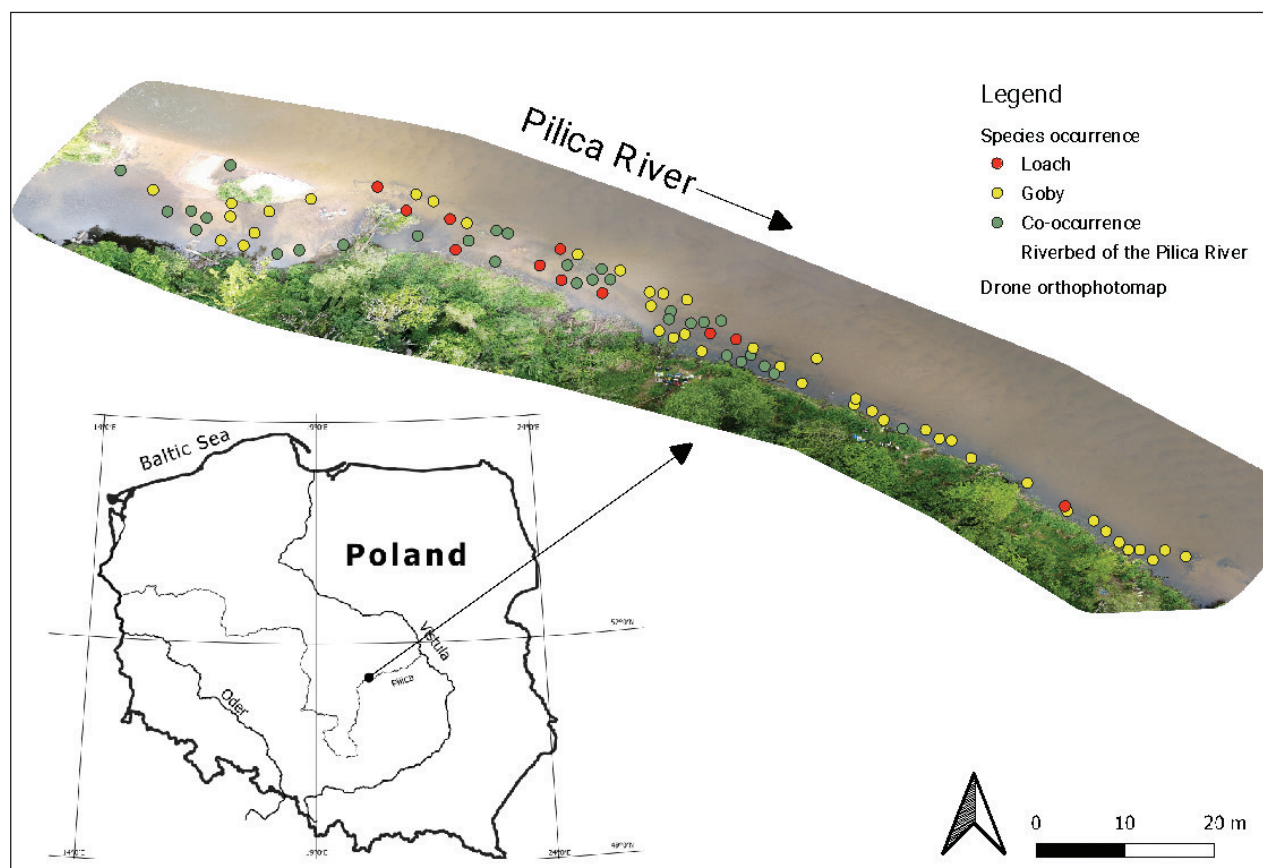


Figure 1. Map of the sampling site located on the Pilica River, where monkey goby (*Neogobius fluviatilis*) and spined loach (*Cobitis taenia*) were collected in May 2024.

species, which burrow in sediment and require time to dig out to escape while electrocuted. Collected fish were placed in buckets and the point of collection was marked. At the bank, individuals were counted and measured to the nearest 1 mm. The whole sampling area with marked points was photographed using a drone (Fig. 1). At each point, a sample of substrate was also taken using a tubular sampler with a cross-sectional area of 25 cm², which was inserted into the sediment to a depth of 15 cm. All collected substrate samples were dried in the lab and sifted (mechanical sieve shaker LAB-11-200) providing data on the percentage of particular substrate fractions (from 128 to 0.063 mm) in individual microhabitats of each fish. In total, 40 specimens of both species (20 monkey goby and 20 spined loach) were taken to compare diet and trophic ecology. All fish were euthanized using an overdose of clove oil.

Diet composition

All fish collected for the gut content analysis were preserved in 4% formaldehyde solution. They were then measured (total length, TL) to the nearest 1 mm and weighed (W) to the nearest 10 mg (monkey goby: 77 ± 22 mm; spined loach: 79 ± 14 mm, on average ± SD). Gut contents were weighed to the nearest 1 mg and stored in glycerin. Food items were subsequently identified to the lowest possible

level of taxonomy; i.e. to order, family or species and/or genus where possible, under a stereomicroscope (Nikon SMZ1000, Japan) and counted. The total number and weight of each prey type were estimated for each fish. The analysis of the diet was based on the percentage of biomass of each prey (%W_i). Prey items were combined by taxon and quantified by the frequency of occurrence (%FO_i) and percentage of biomass (%W_i) (Hyslop 1980). For each food category, the index of importance (IRI) was calculated (Pinkas 1971) and its standardized value (%IRI) (Cortés 1997) was estimated as:

$$IRI_i = \%FO_i \times \%W_i$$

$$\%IRI_i = 100 IRI_i / \Sigma IRI_i$$

where IRI_i is the IRI value for the ith prey category and ΣIRI_i is the total IRI for all prey categories.

To estimate diet overlap, the Schoener α index was used. This index was calculated as:

$$\alpha = 1 - 0.5 \Sigma |p_{ix} - p_{iy}|$$

where p_{ix} and p_{iy} are the biomass proportions of the ith food resource used by monkey goby and spined loach. The Schoener α is the most commonly used niche overlap measure. Values of the index lie between 0, indicating no overlap, and 1, when diets are identical, whereby overlap values exceeding 0.6 are regarded as high or biologically significant (Wallace 1981). For all indices, average values and their standard errors were obtained using the jackknife technique (Krebs 1999).

Stable isotope analysis

Ten specimens of each species were used for stable isotope (SI) analysis (mean TL 92 ± 26 mm and 81 ± 10 mm for monkey goby and spined loach, respectively). Specimens for SI analysis were preserved in ice and stored at -20°C before defrosting. A sample of dorsal muscle tissue was excised from each individual for bulk carbon and nitrogen stable isotope analysis (SIA). White muscle tissue, which has a lower variability in nitrogen isotopic signature compared to other tissues, does not require acidification to remove inorganic carbonates (Pinnegar and Polunin 1999). The sampled muscles were then dried in an oven at a constant temperature of 60°C for 24 hours before being ground to a fine powder using an agate pestle and mortar. Samples of $1 (\pm 0.1)$ mg of homogenized tissues were subsequently analyzed using a Thermo Finnigan Delta Plus Advantagean isotope ratio mass spectrometer at the Biological and Chemical Research Centre in Warsaw, Poland. The isotope compositions were expressed in δ notation (‰), calculated as $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000$, where R represents the $^{13}\text{C}:^{12}\text{C}$ or $^{15}\text{N}:^{14}\text{N}$ ratios. The standards used were Vienna Pee Dee Belemnite for carbon and atmospheric N_2 for nitrogen. To ensure the reliability of the isotopic analysis, samples were analyzed in duplicates. The average standard errors were 0.03‰ for $\delta^{13}\text{C}$ and 0.11‰ for $\delta^{15}\text{N}$.

Laboratory experiment

In autumn 2022, monkey goby and spined loach individuals were collected from the same site as used for the field sampling. The fish were transported to the laboratory in aerated containers. They were then placed in 70-liter aerated aquaria with a water temperature maintained at 17–18 °C. The aquaria were connected to a water circulation system. Each aquarium was equipped with shelters made from 5 cm long PVC pipe halves and artificial plants. The bottom was covered with a thin layer of sand. The number of fish per aquarium ranged from 5 to 8, with more shelters than fish to prevent competition outside the experimental arena. The fish were grouped by species and then by size to ease further matching of individuals for experiment (mean TL 102 mm and 92 mm for the monkey goby and spined loach, respectively). Every other day, they were fed frozen chironomid larvae. The photoperiod was set to 10 hr of light and 14 hr of darkness to mimic natural light conditions during that time. Both stocking aquaria and the experimental tanks were located in the same laboratory room.

Plastic containers (IKEA, Samla) with a capacity of 15 liters (39 × 28 × 14 cm) were used to conduct the experiments. The containers were lined with black plastic wrap to limit the access of stimuli to the experimental arena (also preventing visibility of individuals in adjacent tanks). The containers were divided in half. Approximately 3–4 cm layer of substrate was added at the bottom. Above each container, a camera was installed to record fish behavior (Gemini Technology, GT-CH21C5-28VFW). The water was aerated before starting the experiment and changed before each trial (aerating stones were removed during the experiment).

The experiments were performed in January/February 2023. At first (Experiment 1), we assessed species preferences towards three substrates: fine sand (grain diameter 0.125–0.250 mm), coarse sand (0.5–1 mm) and granule (2–4 mm). Single fish were exposed to two types of substrates (three different treatments: fine sand vs. coarse sand, coarse sand vs. granule). Fish were observed for 20 h and time spent buried in the particular substrate or on exploration (swimming in the tank / not buried) was measured. Those observations enabled us to designate substrate preferred by the majority of individuals (i.e. coarse sand, see the results) as well as avoided one (granule), which were then used in the competition experiment (see below). Each treatment was replicated 10 times.

To evaluate interactions between the monkey goby and spined loach, both species were subject to the following experimental protocol (Experiment 2). The experimental arena was the same as in Experiment 1, with one half filled with coarse sand (preferred substrate, hereafter referred to as “sand”) and the other with granule (avoided, see the results of Experiment 1). A single fish was placed into an experimental tank and given 24 h for acclimation. Then, an intruding fish was introduced (making the first individual a resident) and both individuals were recorded for 20 h, to note their behavior (buried / exploring / aggressive). We tested all species combinations (spined loach vs. spined loach; spined loach vs. monkey goby; monkey goby vs. monkey goby; monkey goby vs. spined loach; resident vs. intruder, respectively), each replicated 10 times. Specimens were used only once in the competition experiment, however, as the spined loach is partially protected by law in Poland, we had to re-use individuals from the preference experiment. All actions were approved by the Local Ethic Committee (52/ŁB251/2022) and the General Directorate of Environmental Protection (WPN.672.8.2022.AGr and WPN.6401.136.2024.BWo).

Data analysis

Previous studies, including Kakareko et al. (2016), have shown that the impact of non-native gobies, such as the racer goby, varies with fish size, with smaller individuals experiencing more adverse effects. Based on this, and to avoid potential biases from pooling all individuals into a single group, we divided both monkey gobies and spined loaches into two groups: small (below 70 mm) and large (70 mm or above). This categorization was informed by the size distribution of fish collected at the site and supported by available literature (e.g. Kakareko 2011; Płachocki et al. 2020).

To identify the most important drivers of habitat use of monkey goby and stone loach in the wild, we used a random forest model with the "rfsrc" function from the randomForestSRC R package. Random forest was selected due to its effectiveness in handling multiple predictor variables and complex interactions without requiring strict parametric assumptions (Breiman 2001), making it well-suited for identifying key drivers in habitat use studies (Kurtul et al. 2024). The dependent variable in this analysis was the abundance of each species, as measured at each sampling point. Individual sampling points served as replicates in the model, allowing us to capture the variation in habitat use across locations. Variable importance in the random forest (RF) was determined by measuring the decrease in prediction accuracy when each variable was randomly permuted, keeping all other variables constant (i.e. a greater decrease in accuracy indicates a more important variable). We used a total of 2,000 decision trees and five nodes in each tree. The out-of-bag (OOB) error rate, which measures the model's predictive accuracy, was 0.12, with an out-of-bag fit value 0.1, indicating a reasonably robust model. To interpret the influence of each predictor on habitat use, we considered the variables mentioned in Fig. 2 and evaluated their impact on species abundance.

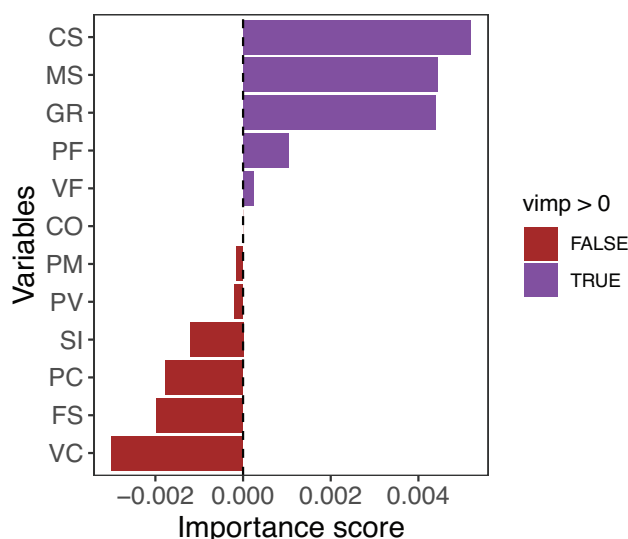


Figure 2. Relative variable importance of habitat type assessed by the applied random forest for affecting the presence of monkey goby (*Neogobius fluviatilis*) and spined loach (*Cobitis taenia*). The variables considered are as follows (particle size ranges in mm): CS (coarse sand, 1–0.5), MS (medium sand, 0.5–0.25), GR (granule gravel, 4–2), PF (fine pebble, 16–8), VF (very fine sand, 0.125–0.063), CO (cobble, 128–64), PM (medium pebble, 32–16), PV (very fine pebble, 8–4), SI (silt, < 0.063), PC (coarse pebble, 64–32), FS (fine sand, 0.25–0.125), and VC (very coarse sand, 2–1). Purple indicates positive effects, while red indicates negative effects. VIMP refers to Variable Importance.

Following on the results of the random forest analysis, which identified the primary substratum (i.e. coarse sand) as the most important predictor (see the results), we applied a Zero-Inflated Negative Binomial (ZINB) model to assess potential interspecific interactions between monkey goby and spined loach using the “glmmTMB” function from the glmmTMB R package (Brooks et al. 2017). In each model, the response variable was the count of either small or large individuals from both goby and loach species. Fixed effects included the counts of the other fish groups (small goby, large goby, small loach, large loach) and the presence of coarse sand (selected based on the random forest analysis results) to assess potential competitive interactions and differences in habitat preferences among species. The ZINB model was chosen due to the high frequency of zero counts in the data and the over-dispersion of fish counts. This model allowed us to explore how the abundance of small and large individuals of each species were influenced by coarse sand as a habitat feature. Before fitting the model, we conducted a thorough data exploration following the guidelines of Ieno and Zuur (2015). This process includes checking for missing values, identifying outliers in both response and explanatory variables, assessing homogeneity and zero inflation in the response variable, evaluating collinearity among explanatory variables, ensuring balance within categorical variables, and examining the relationships between the response and explanatory variables. All candidate models were validated using the DHARMa package in R (Hartig and Lohse 2022). The “simulateResiduals” function was used to simulate standardized residuals, allowing us to check key model assumptions by examining residual patterns for potential deviations (such as homogeneity of variance, normality, and outliers). Additionally, the “plotQQuinif” function generated a Q-Q plot to further assess residual normality. Tests for model dispersion and outliers were also performed during this validation step.

To analyze the differences in the diet between fish species, one-way permutation analysis of similarity (ANOSIM, Bray-Curtis similarity index) was used. The significance level of the R statistics was calculated using 9999 permutations of the dataset. Then, similarity percentage procedure (SIMPER) was applied to distinguish which prey taxa had the greatest contribution to the dissimilarity of the diet of investigated fish species. All multivariate techniques for analyzing diet data were conducted using the PAST v3.15 software (Hammer et al. 2001).

To assess the overlap between the isotopic niches of the two species, we used three complementary approaches. First, we identified if the occupied trophic niches were significantly different using a permutational univariate analysis of variance (PERANOVA) on the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of the two species, with Euclidean distance and 9,999 permutations using the “adonis2” function implemented in the R package vegan (Oksanen 2012). Then, the ratio between the overlap area and the sum of both niche areas for the 95% (maximum likelihood and Bayesian ellipses- SEA_B) and corrected standard ellipse areas for the 40% (SEA_C) standard ellipse areas (SEA) were calculated using the R package SIBER (Jackson et al. 2011). Finally, the directional probability of an individual of a species to occur within the niche of the other species (considered as the 95% and 40% standard ellipse area) was estimated applying a Monte Carlo estimation (chain-length: 10,000 steps) using the R package nicheROVER (Swanson et al. 2015).

To check substratum selectivity in Experiment 1 and 2, we compared percentages of time spent by the fish in coarse sand to a theoretical value of 50% (assuming no selectivity) using one sample Wilcoxon tests. We used a General Linear Model

(GLM) to test the effect of (1) fish species, (2) substratum configuration (coarse & fine sand vs coarse sand & gravel) and (3) time (day vs night, within-subject factor) on percentage of time spent by single fish in movement (proxy for fish activity) in Experiment 1. We distinguished between day and night to account for the temporal factor, acknowledging that both species exhibit variable diel activity patterns (Grabowska et al. 2009; Błońska et al. 2016; Jażdżewski 2020).

We tested factors affecting fish behavior in Experiment 2: (1) percentage of time spent in sand and (2) percentage of time spent in movement using a set of GLMs, separately for each species. First, we tested the effect of (1) intruder presence and (2) intruder species using the measurements of single fish later becoming residents exposed to the presence of intruders. This analysis allowed us to check if the fish respond to the introduction of an intruder and whether this response depends on the intruder species. Second, we compared the behavior of intruders depending on (1) resident species to which they were exposed. This analysis allowed us to check if intruders behaved differently depending on the species of the resident individual. Third, we compared the behavior of fish within single-species pairs depending on (1) individual status (resident vs intruder). This analysis allowed us to check if intruder individuals behaved differently than resident fish staying in the arena for a longer time. In the above models, intruder presence and individual status were modelled as within-subject factors, as measurements were taken twice for the same individual (without and with the intruder), or for two individuals exposed together (resident and intruder), respectively. These models additionally included time (day vs night, within-subject factor) and relative size (resident/intruder length ratio) to control for their effects.

Initial models included all main effects and their interactions. Then, non-significant higher order interactions were dropped from the model in a simplification procedure. As needed, sequential Bonferroni corrected Fisher LSD tests were used as a post-hoc procedure to disentangle significant effects in the models.

Results

Field research

The random forest identified that sand (coarse and medium), granule and fine pebble were the most important variables in predicting presence of monkey goby and spined loach (Fig. 2).

Based on the results of random forest, coarse sand was the primary habitat (Fig. 2). The GLM models indicate that the tested substrate type, as well as conspecifics or non-native goby of different sizes did not have a significant effect on the counts of large and small loach (Table 1), suggesting that other factors may be influencing their habitat use. The marginally non-significant effects of the substrate type on large loach and for small goby on small loach count warrant further investigation (Table 1C, D, respectively).

Diet composition

The analysis of alimentary tract contents showed that, among 19 food categories, the monkey goby fed primarily on Ephemeroptera, Chironomidae, Trichoptera, and *Asellus aquaticus*, complemented by Bivalvia. In turn, the spined loach exploited mainly Chironomidae, Trichoptera, and Simuliidae. Their gut also contained

sand, which was probably consumed additionally with other prey types (Suppl. material 1: table S1). The remaining food categories identified in the diet can be considered as rarely chosen on the basis of their amount and frequency in the diet (Suppl. material 1: table S1). IRI values also indicated that the most important prey for monkey goby were Ephemeroptera, Chironomidae, Trichoptera and *Asellus aquaticus* (48.2%, 21.9%, 12.4%, 12.1% IRI, respectively), what constituted 94.6% IRI in total. In the case of spined loach, Chironomidae, Trichoptera achieved the highest IRI values (46.9%, 25.2%, respectively). The remaining prey categories had lower, equal shares in the fish diet (Suppl. material 1: table S1).

The diet composition and importance of food items differed markedly between fish species (ANOSIM: R-statistic = 0.276, $p < 0.0001$). SIMPER analysis showed that dissimilarity in the diet composition of fish sampled was based on Ephemeroptera, Chironomidae, Insecta remains, *Asellus aquaticus*, Trichoptera and Bivalvia (Table 2). These five categories together constituted over 77.4% of cumulative dissimilarity in the diet between fish species.

Both fish species consumed a wide spectrum of prey groups, but the Schoener α index (0.49 ± 0.039) showed no distinct diet overlap.

Table 1. GLMM analysis of the effect of substratum type and co-occurring fish on the counts of the studied fish species and size classes.

Response variable	Fixed factors	Log-mean	95% CI	P
A. Large goby count	Intercept	-0.78	-1.92–0.37	0.184
	Substrate (sand)	0.01	-0.01–0.03	0.511
	Small goby	-0.07	-0.36–0.23	0.657
	Large loach	0.03	-0.26–0.33	0.823
	Small loach	-0.05	-0.50–0.40	0.826
B. Small goby count	Intercept	0.55	-0.19–1.29	0.146
	Substrate (sand)	-0.01	-0.02–0.01	0.225
	Large loach	-0.19	-0.42–0.05	0.125
	Small loach	0.18	-0.09–0.45	0.180
	Large goby	-0.04	-0.29–0.21	0.753
C. Large loach count	Intercept	0.63	-0.57–1.82	0.304
	Substrate (sand)	-0.02	-0.05–0.00	0.068
	Small goby	-0.25	-0.57–0.07	0.124
	Small loach	0.27	-0.09–0.63	0.142
	Large goby	0.11	-0.22–0.43	0.513
D. Small loach count	Intercept	-1.37	-3.05–0.31	0.110
	Substrate (sand)	-0.01	-0.04–0.02	0.628
	Small goby	0.30	-0.02–0.62	0.065
	Large loach	0.29	-0.08–0.67	0.127
	Large goby	-0.12	-0.64–0.41	0.670

Table 2. Taxa contributing considerably (>5%) to the dissimilarity in diet between the monkey goby (*Neogobius fluviatilis*) and spined loach (*Cobitis taenia*) obtained from SIMPER analysis.

Food category	Dissimilarity		
	Average	Contribution %	Cumulative %
Ephemeroptera	21.73	25.30	25.30
Chironomidae	16.33	19.01	44.31
Insecta remains	8.72	10.16	54.47
<i>Asellus aquaticus</i>	7.94	9.24	63.70
Trichoptera	6.69	7.79	71.49
Bivalvia	5.10	5.93	77.43

Stable isotope analysis

The isotopic niches of the monkey goby and loach were statistically differentiated ($\text{pseudo}F_{1,19} = 23.97$, $P < 0.001$ for $\delta^{13}\text{C}$ and $\text{pseudo}F_{1,19} = 6.09$, $P < 0.02$ for $\delta^{15}\text{N}$), indicating that there was no strong competition between the species. In terms of the 95% Bayesian standard ellipse area (SEA_B), the overlap of the monkey goby with loach was 17.2%. Monkey goby exhibited a wider SEA_B (Fig. 3) and isotopic metrics (Suppl. material 1: table S2) compared to loach. Considering SEA_B , the potential directional overlap of monkey goby with loach was 35.9%, whereas it was 28.9% when considering loach overlapping monkey goby. When considering the 40% corrected standard ellipse areas (SEA_C), the overlap potential was much lower. Monkey goby had a small overlap with loach (5.2%). The probability of loach overlapping monkey goby was 2.4%.

Laboratory experiment

In Experiment 1, single individuals of the spined loach selected coarse sand over fine sand and granule (Fig. 4A, Table 3). The monkey goby preferred sand over granule but did not discriminate between two types of sand. The spined loach were more active than monkey goby (Suppl. material 1: fig. S1A). Moreover, the goby were more active at night than in daylight, whereas the same tendency for the loach was non-significant, resulting in a significant species*time interaction (Suppl. material 1: table S3).

In Experiment 2, the spined loach always occupied an exclusively sandy substratum, irrespective of their status (single, resident, intruder), the presence and species of the accompanying individual and time of the day (Fig. 4B, Suppl. material 1: table S4). In most cases, the monkey goby also exhibited such preference, except intruder gobies in single-species configurations and intruder gobies facing the spined loach in daylight (Fig. 4B, Suppl. material 1: table S4).

Due to the lack of variability in sand occupation by the spined loach, we ran models testing this variable only for the monkey goby data. The introduction of an intruder, irrespective of its species, increased the time spent by the monkey goby in the sandy substratum (Fig. 5A, Table 4A). Goby intruders exposed to spined loach residents at night spent more time in the sandy substratum than in daylight and in the presence of conspecific residents (Fig. 5B) as shown by a significant resident species*time interaction (Table 4B). In single species pairs, resident gobies spent more time in the sandy substratum than intruder individuals (Table 4C, Fig. 5C).

The spined loach were more active in the presence of conspecific intruders than in the presence of gobies (Suppl. material 1: fig. S1B, table S5A), as well as at night than in daylight (Suppl. material 1: fig. S1C). Moreover, in daylight, they reduced their activity when an intruder was introduced to the tank, as shown by a

Table 3. Substratum preferences of single spined loach (*Cobitis taenia*) and monkey goby (*Neogobius fluviatilis*) in Experiment 1. One sample Wilcoxon tests comparing percentages of time spent by the fish in coarse sand to a theoretical value of 50% (assuming no selectivity).

Species	Substrata	Z	P
Spined loach	Fine vs coarse sand	-2.46	0.014*
Spined loach	Coarse sand vs granule	-3.16	0.002*
Monkey goby	Fine vs coarse sand	-0.05	0.958
Spined loach	Coarse sand vs granule	-2.27	0.023*

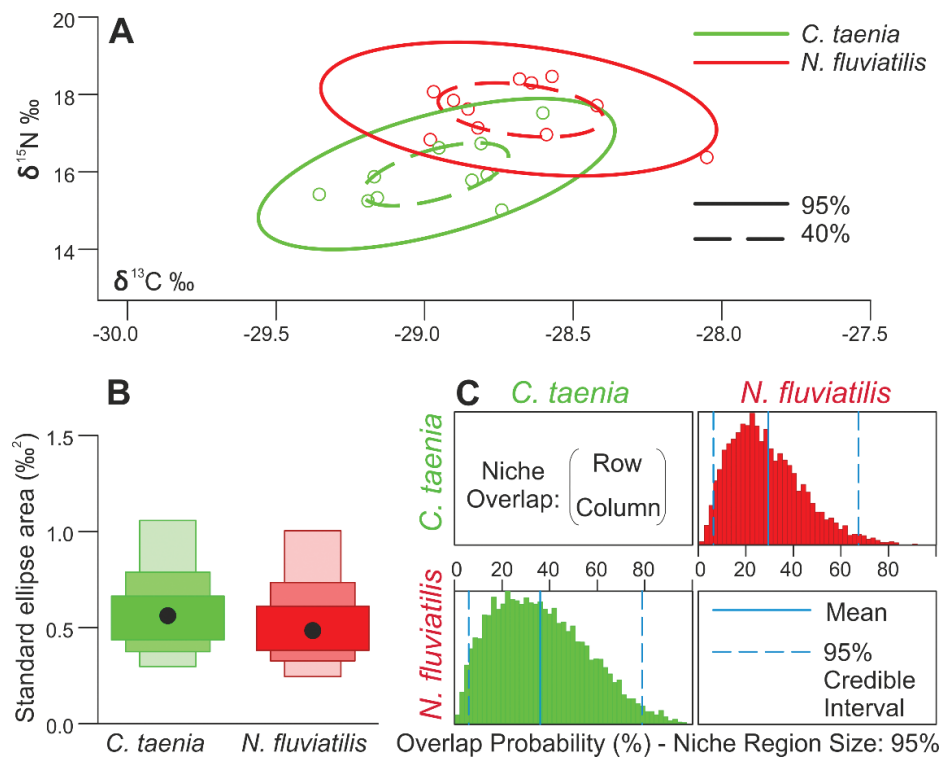


Figure 3. A Standard Ellipse Areas (SEA) for spined loach (*C. taenia*) and monkey goby (*N. fluviatilis*): 95% (solid lines) and 40% (dashed lines) B A posteriori distributions for the Bayesian standard ellipse areas (SEA_b) C Niche overlap based on the 95% confidence interval (SEA_b).

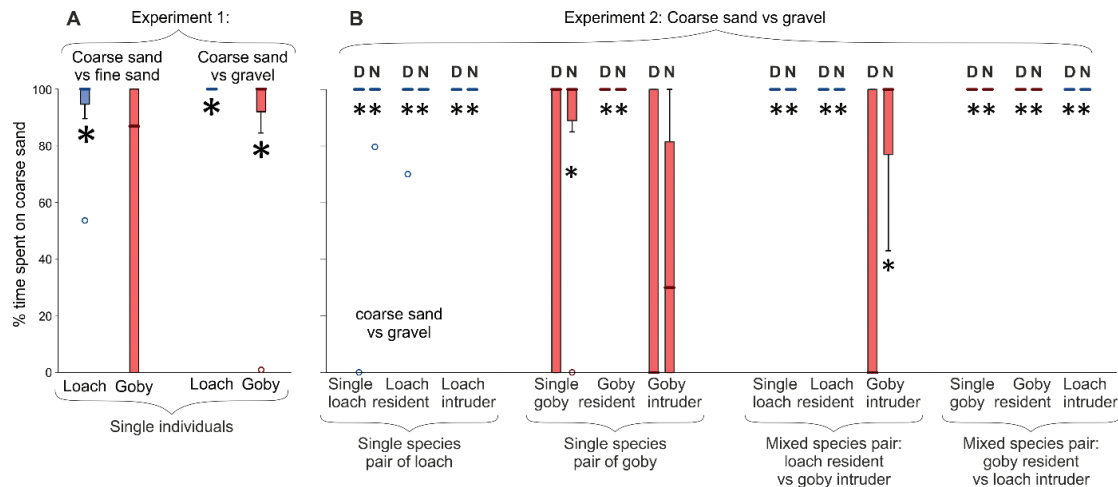


Figure 4. Substratum preferences (percentage of time spent by fish on coarse sand in the presence of alternative substratum) of spined loach (*C. taenia*) and monkey goby (*N. fluviatilis*) A Preference of single fish exposed for coarse sand vs fine sand or granule in Experiment 1 B Preferences of fish in single-species and mixed species pairs in Experiment 2, depending on their status (single fish, residents in the presence of intruders, intruders in the presence of residents). Values differing significantly from 50% (indicated by asterisks, see Table 3 and Suppl. material 1: table S5 for panel A and B, respectively) indicate preferences for coarse sand. Horizontal lines, boxes, whiskers, and circles indicate medians, 1st and 3rd quartiles, 1.5* interquartile ranges and outliers, respectively.

significant intruder presence*time interaction (Suppl. material 1: table S5A). The monkey goby reduced their activity when an intruder was introduced to the tank and were more active in the presence of spined loach vs conspecific intruders, as well as at night vs in daylight (Suppl. material 1: table S5B, fig. S1E–G). The activity of intruder fish of both species was not affected by the resident species. They were more active at night than in daylight (Suppl. material 1: table S5C, D).

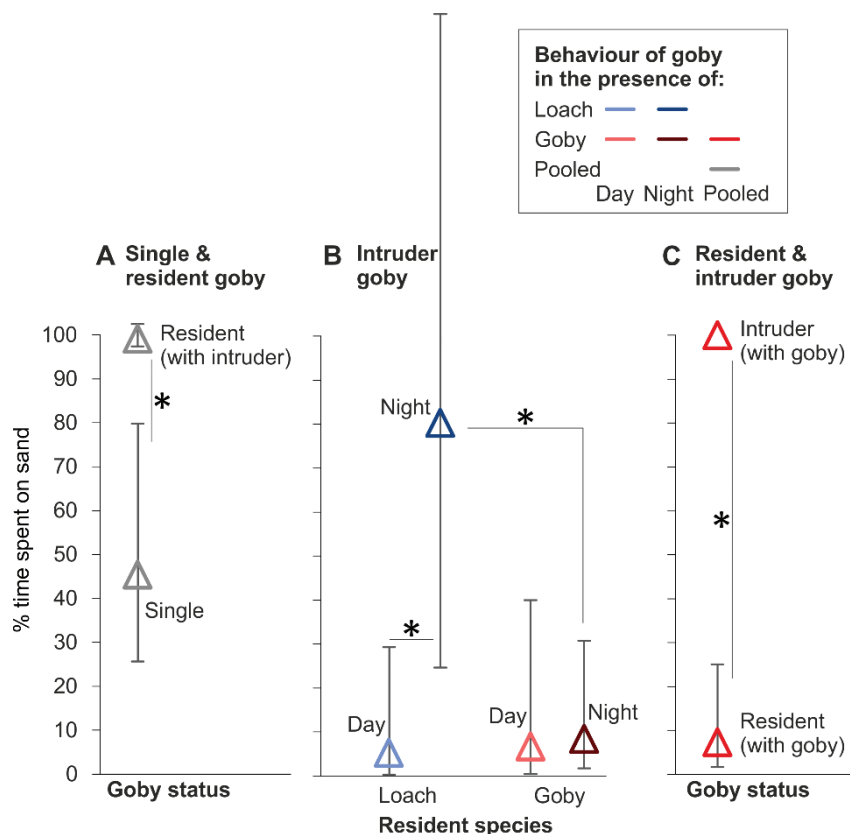


Figure 5. Time spent on coarse sand by monkey goby (*Neogobius fluviatilis*) in Experiment 2 in single-species and mixed species pairs, depending on their status (single fish, residents in the presence of intruders, intruders in the presence of residents) and time of the day (presentation of significant effects from the models reported in Table 4) **A** Behavior of gobies tested as single fish vs. behavior of goby residents after the introduction of intruders **B** Behavior of goby intruders tested in different times of the day in the presence of various resident species **C** Behavior of residents vs. intruders in single-species pairs. Data are presented as means predicted by the models \pm 95% CI. Asterisks indicate significant differences. The spined loach always spent 100% of time on coarse sand, irrespective of their status, accompanying species, and time of the day.

Table 4. Substratum selection (percentage of time spent in sand) by monkey goby (*Neogobius fluviatilis*) in single and mixed species pair in Experiment 2. General Linear Models to test **A** Effect of intruder presence and species on behavior of single/resident individuals **B** Effect of resident species on behavior of intruders **C** Differences in behavior between residents and intruders in single-species pairs. The models additionally included time (day vs night) and relative size (resident/intruder length ratio) to control for their effects. Non-significant higher order interactions dropped from the model in a simplification procedure.

	Comparison	Effect	F	df	P
A	Single vs resident fish (with different intruders)	Intruder presence ^{WS}	7.64	1, 70	0.007*
		Intruder species	0.00	1, 70	0.997
		Time ^{WS}	3.40	1, 70	0.069
		Relative size	0.00	1, 70	0.999
B	Intruders with different residents	Resident species (RS)	1.45	1, 31	0.238
		Time (T) ^{WS}	5.44	1, 31	0.026*
		RS*T	4.59	1, 31	0.040*
		Relative size	0.01	1, 31	0.923
C	Resident vs intruder within each pair	Individual status ^{WS}	19.77	1, 33	<0.001*
		Time ^{WS}	0.00	1, 33	1.000
		Relative size	0.00	1, 33	1.000

^{WS} – within-subject factor (repeated measures for the same fish of pair of fish).

Spined loach intruders were more active than residents in daylight, but not at night (Suppl. material 1: fig. S1D), as shown by a significant individual status*-time interaction (Suppl. material 1: table S5E). The goby intruders were always more active than residents (Suppl. material 1: fig. S1H, table S5F).

Discussion

Our current study showed that both species, the monkey goby and spined loach, occupied the same habitats at the site of their co-occurrence, their diet composition did not considerably overlap and clearly differentiated isotopic niches also indicated lack of strong food competition. The lack of negative impact of monkey goby on spined loach was additionally demonstrated in experimental condition under the limited resource choice as both species co-occupying preferred substrates and monkey goby did not appear to be stronger competitor than conspecific intruders.

Under natural conditions, both species occupied substrates dominated by coarse and medium sand fractions (1–0.5 and 0.5–0.25 mm, respectively). Most studies on the monkey goby habitat preferences have demonstrated its affinity for sand (Grabowska et al. 2023); however, there are no studies evaluating preferences towards specific substrate fractions (grain diameter), either under natural or experimental conditions. The high association of monkey goby with sand substrate is correlated with its morphology (relatively small head and ventral lobe), leading to strong specialization towards burrowing into soft substrate (Čáková et al. 2008; Grabowska et al. 2023). This habit is believed to help in avoiding predators (Holčík et al. 2003), however, it is also suggested that their strong preference for sandy substrates could limit the expansion of their invasive range (Čáková et al. 2008; Piria et al. 2016). Nevertheless, a significant proportion of sand is associated with high abundances of the species and can be used as a key determinant for its presence (Płachocki et al. 2020; Grabowska et al. 2023). Association of the spined loach with fine-grain substrate has been confirmed in various studies, as well as by its burrowing behavior (e.g. Robotham 1978; Slavík et al. 2000; Pietraszewski 2015). Detailed studies on the spined loach habitat preferences were conducted by Pietraszewski (2015) at the sampling site located in the same river close to our sampling location, but without monkey goby. The species occupied microhabitats dominated by fine and very fine sand and silt (0.250–0.125, 0.125–0.063, < 0.063 mm, respectively) with reduced presence of very coarse sand and granules (1–2 and 2–4 mm, respectively). These results were not confirmed in our study, in which fine and very fine sand and silt constituted less than 2.5% of the substrate, while very coarse sand and granules made up 27% (combined). Among all evaluated points, 39% (37 out of 95) were occupied by both monkey goby and spined loach and there was no significant effect of monkey goby presence on spined loach, regardless of fish size, which suggests the lack of intensive competitive interactions for microhabitats. Čáková et al. (2008) suggested that among the round, bighead, and monkey goby, the last species would have the least impact on native communities. In the Sava River, the presence of monkey gobies initially indicated a decrease in the proportion of gudgeon *Gobio gobio*, suggesting a potential adverse impact (Jakovlić et al. 2015). However, subsequent studies conducted in the same river and similar locations did not confirm these findings (Piria et al. 2016).

In addition to our field results, we carried out laboratory experiments to explore species interactions, specifically examining their habitat selection behaviors. Trials conducted individually showed that spined loach preferred coarse sand over finer and coarser materials, which complements our field observations.

The monkey goby displayed a higher flexibility, not discriminating between fine and coarse sand, while both species avoided granule substrates. In competition trials, there was no observed effect of the monkey goby on spined loach. Both species continued to avoid granule substrates and co-occupied the limited coarse sand patches, indicating no direct competitive interactions between them.

We observed a reduced activity of resident fish compared to their behavior as single individuals immediately after putting them in the experimental tanks. Moreover, intruder fish were more active than resident individuals. This points to the interpretation of increased activity as a response to an environmental stressor (here: a new locality and the presence of another, already established individual). In the case of the spined loach, this hypothesis is also supported by the fact that the above-mentioned differences were exhibited in daylight, where potential environmental dangers are stronger and increased exploration is not likely to be beneficial. Therefore, the higher activity of resident fish (of both species) in the presence of intruding spined loach vs monkey goby, irrespective of the time of the day, suggests that the former intruder poses a stronger negative effect on the behavior of both conspecifics than monkey goby. This is likely to result from the higher general activity of the spined loach in our study. Moreover, only intruding monkey goby moved from their normally preferred sandy substratum to avoided gravel in the presence of conspecifics and spined loach (only in daylight) residents, whereas the substratum selection by the spined loach remained unaffected. Therefore, these results suggest that the presumably invasive monkey goby does not exert a strong effect on coexisting spined loach, affecting their behavior to a lower extent than the presence of conspecific loach. Previous studies suggested no diel pattern in monkey goby activity (Grabowska et al. 2009). Spined loach is usually considered to be more active during the night, but this has been questioned and remains unconfirmed by certain studies (Jażdżewski 2020). Previous laboratory studies have also found the monkey goby to be a benign interference competitor (Van Kessel et al. 2011). Błońska et al. (2016) noted additionally that the influence of the monkey goby on the counterpart species was comparable to that of another conspecific.

Results of our study at the site of both species' co-occurrence confirmed their broad diet spectrum. Monkey goby consumed mostly Ephemeroptera, Chironomidae, Trichoptera larvae and *Asellus aquaticus*, while Chironomidae, Trichoptera and Simuliidae larvae dominated the diet of the spined loach. However, although some prey categories were found in the guts of both species, their relative importance in the diet was different and no distinct diet overlap was observed. The dietary habits of the monkey goby and the spined loach reveal the lack of specialization in prey selection (Jażdżewski 2020; Grabowska et al. 2023).

The trophic ecology of both species indicated a higher trophic position for the monkey goby compared to spined loach, with prey item overlap of only 17%. This finding supports the results of the stomach content analysis. A flexible, opportunistic feeding strategy with a variety of prey items included seems to allow the co-occurrence of monkey goby and spined loach without negative effects.

Conclusion

When considering non-native species, the most common expectation is their negative impact on recipient communities, which has been supported by numerous studies (Cucherousset and Olden 2011; Britton 2023). However, not all non-na-

tive species pose a similar threat. The research often focuses on cases where abundant invaders lead to significant ecological changes, potentially overstating the impact of non-native species in general (Gozlan 2008; Jackson et al. 2015). Therefore, it is crucial for prioritizing proper management actions (Britton et al. 2023) to report also cases where the impact of non-native species is minimal or relatively benign (e.g., Fobert et al. 2011) or where biotic resistance of native species and ecosystems is considerably high. While analyzing ecological interactions, it is important to consider that spurious correlations can sometimes arise, especially when external factors, such as human impact, simultaneously facilitate invasive species and adversely affect native populations (Tarkan et al. 2012). This may lead to the mistaken assumption that the invasive species directly harms the natives, when in fact, both may be responding independently to the same external pressures. Our comprehensive study showed that, although the monkey goby displays similar diet, habitat, and behavioral patterns as the spined loach, no adverse effect of this non-native species was observed at the studied location. It is possible that habitat partitioning, rather than competition, occurs in this species set, as it has been also observed in other cases involving non-native species (Gjelland et al. 2007; Guo et al. 2012; Kakareko et al. 2016; Grabowska et al. 2024). Despite the monkey goby's increased abundance and extended range, the spined loach population persists (own observ.). Our results do not contradict the invasive character of the monkey goby at other locations and/or in other ecological contexts, e.g. considering other native species or other biological traits. Moreover, the dynamic climate change severely affecting freshwater environments may exert a stronger competitive effect on the spined loach in the future (Fobert et al. 2011). This emphasizes and highlights the importance of context- and site-dependent factors in biological invasions, as the outcomes of interactions between native and non-native species can vary significantly, depending on local environmental conditions and species-specific traits. Such variability highlights and underscores the critical need for site-specific studies to enhance our understanding, prediction, and management of the impacts of invasive species (Błońska et al. 2024).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.







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Author contributions

DB – conceptualization, data curation, investigation, project administration, writing – original draft; KP, JL – data curation, investigation, formal analysis, visualization, writing – review & editing; BJ – data curation, investigation, visualization; JK – formal analysis, visualization, writing – review & editing; JG – writing – review & editing; AST – formal analysis, visualization, writing – original draft.

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Data availability

Raw data used in the manuscript that are not already provided are available from the corresponding author on reasonable request.

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Supplementary material 1

Habitat and diet interactions in a lowland temperate river suggests no direct impact of non-native monkey goby (*Neogobius fluviatilis*) on native spined loach (*Cobitis taenia*)

Authors: Dagmara Błońska, Kacper Pyrzanowski, Joanna Leszczyńska, Bartosz Janic, Jarosław Kobak, Joanna Grabowska, Ali Serhan Tarkan

Data type: docx

Explanation note: Table showing diet composition, and a table and figure on preferences and activity from the lab experiment.

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Research Article

Non-native species drive the global loss of freshwater fish beta-diversity

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Abstract

Freshwater ecosystems are facing mounting challenges. The widespread introduction of non-native species, for example, has resulted in the loss of native species and the substantial reconfiguration of diversity patterns across regions. Documenting such impacts remains critical for informing national-level biosecurity policies. Here, we explore changes in biogeographic patterns in freshwater fish diversity in response to the spread of non-native species, teasing apart the geographic (watersheds) and taxonomic (species) drivers of patterns at the global scale. We leveraged global databases of fish species occurrence to estimate the unique contributions of local watersheds and species (native and non-native origin) to beta-diversity for biogeographic domains. Beta-diversity metrics of watersheds and species at a domain scale can be interpreted as their importance for the uniqueness in freshwater fish composition. We report significant changes in freshwater fish beta-diversity in response to non-native species, with the largest impacts in the Ethiopian, Nearctic and Palearctic domains, even though non-natives decreased the contribution of watersheds to beta-diversity in all domains, particularly in watersheds with known impacts. Watersheds identified as most important for promoting beta-diversity were not evenly distributed across domains, were influenced by geographical isolation and their unique compositions were composed of many endemic and threatened species. Highest values of species contributions to enhancing beta-diversity were mainly observed for native and threatened species, although mean values of species contributions were higher for non-threatened species. Species from the most important watersheds had wide ecological tolerances, were, in general, natives, endemics and/or with IUCN threat status. Our findings underscore the widespread consequences of non-native species for shaping biogeographic patterns of freshwater fishes in the Anthropocene.

Key words: Beta diversity, biodiversity conservation, biogeographic domain, biotic homogenisation, exotic species



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Introduction

Limitations on dispersal ability have produced the interesting phenomenon that many, perhaps even most, species do not occupy all the areas of the world in which they could survive (Darwin 1859). This is perhaps no better demonstrated than in freshwater ecosystems, where high diversity and endemism stem largely from the fact that freshwaters are embedded within a terrestrial landscape that limits

dispersal within and amongst drainage basins (Olden et al. 2010). River basins or watersheds act as “islands” where fish evolution occurs somewhat independently (Tedesco et al. 2012; Su et al. 2019). As a consequence, a complex combination of factors shapes broad-scale diversity patterns of freshwater fish, including the palaeo-connectivity of watersheds, eco-evolutionary processes and environmental variability interacting at different scales (Leprieur et al. 2009; Dias et al. 2014; Carvajal-Quintero et al. 2019).

The regional connectivity of the world is stronger and more varied than ever before. In this sense, human-induced biological invasions have been growing in the last centuries and there is no indication that rates are decreasing to a saturation level (Seebens et al. 2017). It is now a consensus that biological invasions cause impacts not only at local scales, but are also responsible for a global reshuffling of biogeographic patterns (Leroy et al. 2023), leading to biotic homogenisation of ecological communities (McKinney and Lockwood 1999). The widespread introduction of non-native species has dramatically reconfigured patterns of diversity, often leading to the loss of native species (Pyšek et al. 2020; Su et al. 2021) and the dissolving of biological uniqueness across regions (McKinney and Lockwood 1999; Olden 2006). Such homogenising effects are ubiquitous across spatial scales and taxonomic groups, particularly for freshwater fishes (Olden et al. 2018; Padial et al. 2020). Human-mediated dispersal of non-native freshwater fishes occurs as a result of numerous pathways, including aquaculture practices, ornamental pet trade, release of bait for angling, biological control, stocking for fisheries, shipping ballast transport and interconnected waterways amongst others (Bernery et al. 2022). The features that explain successful establishment of non-native species (i.e. invasiveness) together with the characteristics of the receiving environment (i.e. invasibility) interact to produce the patterns of invasions (Skóra et al. 2015; Hui et al. 2016; Xu et al. 2024).

The impacts of non-native species introductions are truly global in scale (Seebens et al. 2017; Capinha et al. 2022) and the homogenisation of freshwater fish faunas in response to non-native species is increasingly recognised [e.g. Leprieur et al. (2008); Olden et al. (2008); Marr et al. (2013); Liu et al. (2017)]. The change is so dramatic that even well-accepted biostratigraphic boundaries for biological communities, known as biogeographic domains, are being re-arranged in the Anthropocene, creating new domains such as the ‘Pan-Anthropocenian Global North and East Asia’ (PAGNEA, *sensu* Leroy et al. 2023). For freshwater fish faunas, such dramatic modifications pose a significant concern (Olden et al. 2010; Cucherousset and Olden 2011), as preserving broad-scale beta-diversity is a priority in large-scale conservation planning (Socolar et al. 2016; Su et al. 2019). At the same time, changes in the fish community may cause impacts on the ecosystem services provided by them, such as the provisioning of food (i.e. fisheries and aquaculture), the regulation of pest controls (i.e. insects), the supporting of nutrient cycling and ecosystem engineering, as well as many cultural services associated with traditional culture and fishery (Pelicice et al. 2023).

The Emergency Recovery Plan required to “bend the curve” in freshwater biodiversity loss explicitly calls for a renewed focus on preventing the impacts of non-native species (Tickner et al. 2020). The recovery plan and its recommendations are aligned with several sustainable development goals and targets of the Kunming–Montreal Global Biodiversity Framework (2022) aiming to restore and recover biodiversity by 2050. Recovery planning must ensure the conservation of native fish biogeography in the light of past and likely future species invasions (Britton et al. 2023). In this sense, describing patterns in beta-diversity studies

is central to better providing information for conservation efforts (Socolar et al. 2016); and a meaningful scale for freshwater fish are watersheds within biogeographical domains [e.g. Tedesco et al. (2017); Leroy et al. (2019)]. For instance, changes in beta-diversity indices amongst watersheds may provide information for the impact of non-native fish species on the biogeographic patterns of continental aquatic environments. Additionally, beta-diversity patterns indicate those watersheds and species that are most important for promoting regional compositional differences and combatting growing trends towards a more homogenised world. For instance, the watersheds and species that mostly contribute to compositional uniqueness in the biogeographic domain would be those deserving conservation efforts to mitigate biotic homogenisation (Xia et al. 2022).

Here, we sought to disentangle the roles of native and non-native species in shaping contemporary patterns of freshwater fish beta-diversity across biogeographic domains of the world. We identify and map watersheds that remain strongholds in enhancing fish beta-diversity and determine those species contributing the most to these patterns. By elucidating the pattern and drivers of changes in freshwater fish beta-diversity, we aim to provide information for national and international policies and conservation strategies that seek to preserve the uniqueness of the world's fish fauna in the light of ongoing species introductions.

Material and methods

Ichthyofauna global database and biogeographic domains

The compositional data by river watershed in the biogeographic domains were obtained from the ichthyofauna database published by Tedesco et al. (2017). The database contains species lists for > 3,000 watersheds covering more than 80% of the Earth's surface and includes nearly 15,000 fish species inhabiting permanently or occasionally freshwaters. The database was based on surveys of 1,436 published papers, books, grey literature and web-based sources (Tedesco et al. 2017). Watersheds were organised according to biogeographic domains that were proposed by Leroy et al. (2019) as meaningful regions for freshwater fish: Australian, Ethiopian, Madagascar, Nearctic, Neotropical, Palearctic and Sino-Oriental. Indeed, Muñoz-Mas et al. (2023) already demonstrated that this classification is suitable to understand global patterns of freshwater fish invasions. We updated the database by omitting extinct species according to the revision by Su et al. (2021) and by classifying non-native species as those originally foreign to the biogeographic domain, but introduced directly or indirectly by humans. Metadata and complete sheets of species occurrence per watershed are freely available for download at <<https://doi.org/10.1038/sdata.2017.141>> and classifications of biogeographic domains are available at <<https://borisleroy.com/fish-biogeography/>>. The Madagascar domain was not used, given the low number of watersheds, making beta-diversity analyses meaningless.

Watersheds were differentiated by exorheic (watersheds having an estuary with an outlet to the sea or ocean) and endorheic (watersheds not having an outlet to the sea or ocean). Species origin was confirmed according to the Global Invasive Species Database – GISD (Pagad et al. 2015) and the Invasive Species Compendium (CABI 2021). Once the database was updated, we used the “fishbase.valid.name” record as the official record of species by watershed. Updates to the current species name or its classification into subspecies were also made when necessary.

Beta-diversity indices

Beta-diversity (β) – a measure of the amount of change in species composition from one location to another (Whittaker 1972) – can be partitioned into unique variations contributed by individual sites and species within the dissimilarity matrix (Legendre and De Cáceres 2013). This method is suitable to identify the watersheds and species that mostly contribute to the heterogeneity and compositional uniqueness of a biogeographic domain. For that, we assessed beta-diversity using the index proposed by Legendre and De Cáceres (2013). This index partitions the total beta-diversity (BDT) into the species contribution to beta-diversity (SCBD) and local (site) contribution to beta-diversity (LCBD); this measure of beta-diversity is estimated independently of local (α) and regional (γ) diversity. We used the *adespatial* package (Dray et al. 2021) to estimate the BDT, SCBD and LCBD indices using the *beta.div* function and the Hellinger transformation of the community matrix for each biogeographic domain. We excluded three watersheds from the analyses that had no native species.

SCBD values represent the relative contribution of species in the study area, interpreted here as the relative contribution of fish species to the total beta-diversity of each biogeographic domain. LCBD values indicate the uniqueness of the river watersheds (sampling units) in terms of the fish composition for each biogeographic domain. In a conservation perspective, species with high SCBD in the domain are those that mostly contribute to compositional variation amongst watersheds and should be prioritised in conservation efforts; and watersheds with high LCBD are those harbouring unique freshwater fish composition, being thus central to mitigate biotic homogenisation amongst watersheds.

Watershed contributions to beta-diversity

Watershed contributions to beta-diversity (LCBD) were mapped across the world according to species of all origin (native and non-native) to represent the “present-day” time-period and according to native species only to represent the “historical” time-period prior to non-native species introductions. In addition, the classification of native and non-native has a time limitation, as information prior to 1850 is difficult to secure. The change in LCBD was due to the introduction of non-native species ($\Delta\text{LCBD} = \text{LCBD}_{\text{present}} - \text{LCBD}_{\text{historical}}$). Positive ΔLCBD values indicate watersheds that have maintained or increased their contribution to beta-diversity in the present-day, whereas negative values indicate watersheds with decreasing contributions due to non-native species introductions. Next, we investigated the relationship between LCBD (and ΔLCBD) and the proportional richness of non-native species. Our expectation is that highly-invaded watersheds will exhibit LCBD decreases over time. We thus used a local polynomial regression fitting method (see Cleveland et al. 1992), which is suitable to investigate an overall trend that is not necessarily linear. We chose this method given our goal was to run an exploratory analysis without establishing a functional relationship. Therefore, trend strengths were described using only the R-squared fit, not p-values. We also investigated the overall trend between LCBD and present-day α diversity (number of fish species) for each watershed. A positive association would indicate that the watersheds most important for beta-diversity would be those with higher species richness. We

then compared LCBD between endorheic (river mouth is not at the ocean) and exorheic (river mouth is at the ocean) watersheds using a Welch two-sample t-test, given the lack of homoscedasticity (standard deviations were always different between groups). The complete database of LCBD values, total species richness and number of non-natives for all evaluated watersheds is available as Suppl. material 1. For all graphs and analyses, LCBD values were standardised (values scaled to zero mean and unit variance) within each domain to facilitate comparisons amongst domains that differed considerably in the number of watersheds and overall species richness.

Species contributions to beta-diversity

SCBD were calculated (and standardised as described for LCBD) for all species in each domain and related to relative species occupancy (%) calculated as the percentage of watersheds occupied by species, using local polynomial regression fitting method (Cleveland et al. 1992) to describe the overall trend, following the same justification as mentioned above for LCBD trends. We explored these relationships per domain separately (Suppl. material 1). Standardised SCBD were compared between species classified into two groups: ‘Least concern’ and those with some threat status (excluding the already extinct species, those not evaluated or those with data deficient) based on the IUCN threat categories of species (*sensu* <<https://www.iucnredlist.org/en>>) using Welch two-sample t-tests also given the lack of homoscedasticity. Finally, we described the five most important species (i.e. the highest SCBD) for each domain according to their origin, endemism, IUCN threat status and the following ecological characteristics (Froese and Pauly 2021):

- i. Habitat: Euryhaline (EU) refers to species that can occupy environments of different salinities, but not necessarily for spawning; Freshwater (FRE) refers to species that occupy freshwater in the entire life cycle and may tolerate only mild estuarine conditions.
- ii. Migration: Non-migratory (NM) species have sedentary behaviour and limited movements along the watershed; Potamodromous (PO) species migrate to spawn in freshwater ecosystems; Catadromous (CA) species live in freshwater and migrate to estuaries/oceans to spawn; Anadromous (ANA) species migrate up rivers from the oceans to spawn; Amphidromous (AM) species migrate from freshwater to saltwater or vice versa at some stage in their life cycle, but not necessarily to spawn.
- iii. Vertical position in the water column: Demersal (DE) refers to species that live near or at the bottom of the aquatic environment; Benthopelagic (BP) species live in the water column or at the bottom of the aquatic environment; Pelagic-neritic (PN) species occur in the water column and near littoral areas.

The complete database of SCBD values for all species, as well as their occupancy and origin (native or non-native) in the domains and the IUCN threat status (if available) is provided as Suppl. material 1. All analyses were performed in the R language (R Core Team 2024), graphs and polynomial regressions were generated using the *ggplot2* package (statistics were obtained using ‘loess’ function; Wickham 2016) and maps used the same watershed definitions, names and acronyms of Tedesco et al. (2017).

Results

Watersheds displayed marked variability in their contributions to fish beta-diversity, with these contributions changing in response to the inclusion of non-native species. We depicted historical LCBD in the left panels, where high values indicate basins with a unique freshwater composition and changes in LCBD due to non-native species in the right panels, with negative values indicating biotic homogenisation and positive values indicating differentiation (Fig. 1). In the Australian domain, the most contributing watersheds are located in Indonesia and Papua New Guinea, as well as in central and east Australia; whereas in west Australia, the watersheds demonstrated a decreased LCBD due to non-native species. In Ethiopia, the highest contributing watersheds are predominantly located in north and west Africa, as well as Middle East, with also small important watersheds in east Africa. Watersheds in south Africa were the ones that mostly changed due to the introduction of non-native species. Nearctic watersheds had LCBD values that varied substantially across space: the most important watersheds were located both in extreme south (mostly) and north of the domain; and those that mostly decreased LCBD due to non-native species are concentrated in the east and (mostly) west of USA and Canada. The most important watersheds in the Neotropic included both small watersheds near the Andes Mountains as well as the world's largest watershed, the Amazon. In this domain, the watersheds that mostly changed due to non-natives were generally also small near the Andes and one in northeast Brazil. Palearctic's most important watersheds were generally located in the southern tropical areas of the domain (except some in north and northeast Russia and small ones near the Mediterranean Region); and the most impacted watersheds include locations in the Middle East, several small watersheds in the Mediterranean Region and also in the UK. Finally, the Sino-Oriental domain had a relatively homogeneous geographic distribution of LCBD, but the most important were rarely in the north-eastern region of the domain. Watersheds with high changes were located in north-west China, Kirghizistan and Kazakhstan, as well as some small watersheds in Japan.

The relationship between the LCBD with and without non-native species is available for all domains in Fig. 2 and for each separate domain in Suppl. material 1. If the exclusion of non-native species does not change the watershed importance to beta-diversity, then the values would fall on the expected dashed line in figures. Patterns revealed those domains with watersheds harbouring more non-native species were Ethiopian, Nearctic, Palearctic and Sino-Oriental (Fig. 2). Accordingly, those were the domains in which LCBDs mostly changed. Watersheds with a high number of non-native species made were those that mostly varied from the expected mean (i.e. no change in LCBD), proportionally decreasing the LCBD with non-natives (i.e. values above the expected mean). Additionally, the watersheds with higher LCBD values were those with a higher proportion of non-native species for Ethiopian, Neotropical and Sino-Oriental domains (Fig. 2, Suppl. material 1). These three domains are those harbouring watersheds with the highest species richness. LCBD for Australian domain watersheds were the less variable (Fig. 2). See also Suppl. material 1 for graphs separated by domain.

By looking at the LCBD changes across domains, only the Australian domain did not have a clear negative relationship between changes in LCBD after removing non-native species and the proportion of non-natives, but the other domains do (Fig. 3).

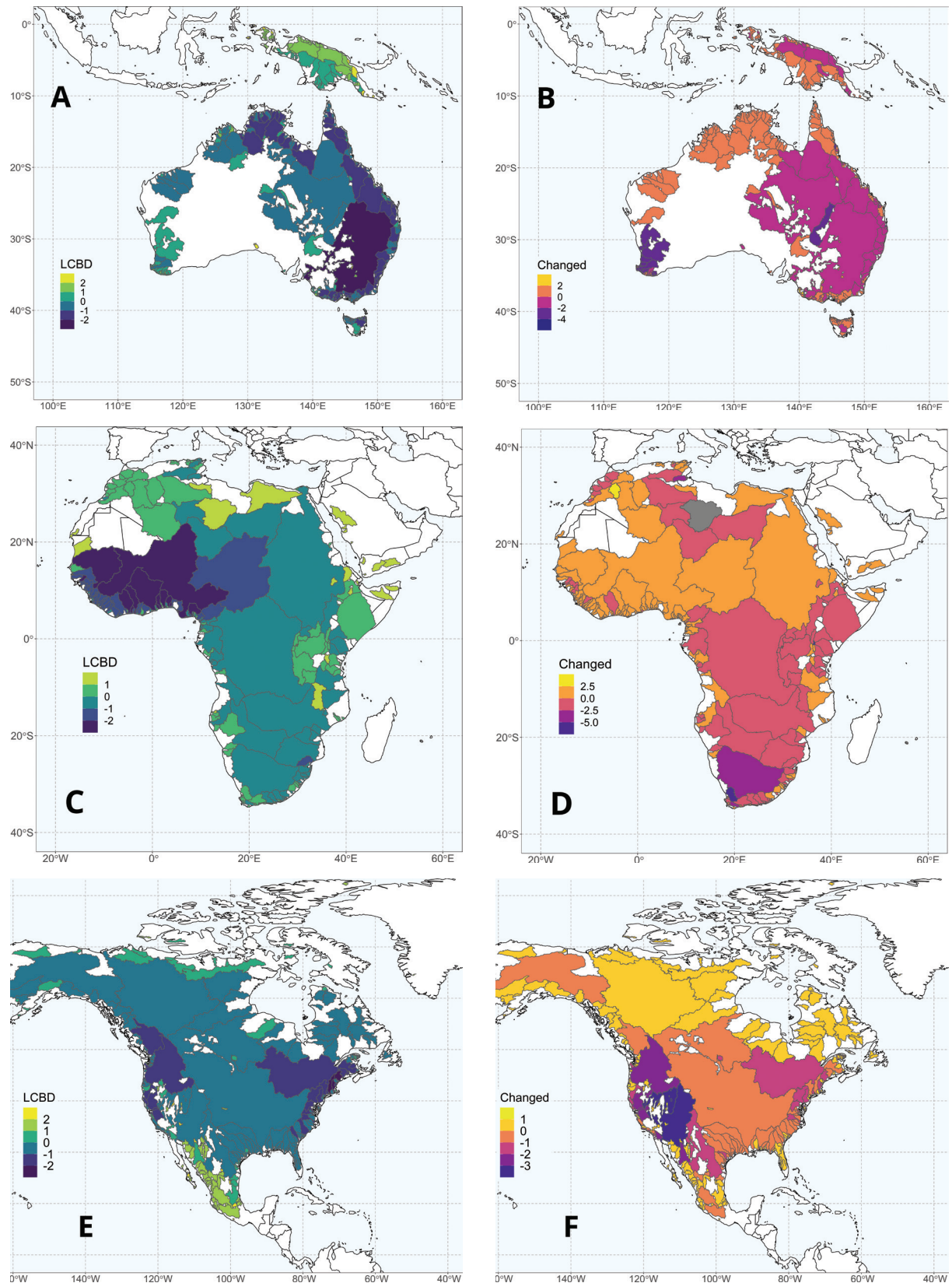


Figure 1. Maps for each biogeographical domain showing the local contribution to beta-diversity (LCBD) of watersheds with non-natives and the decrease in LCBD due to non-native species (Change = LCBD with non-natives – LCBD without non-natives) **A** LCBD – Australian **B** LCBD change – Australian **C** LCBD – Ethiopian **D** LCBD change – Ethiopian **E** LCBD – Nearctic **F** LCBD change – Nearctic **G** LCBD – Neotropical **H** LCBD change – Neotropical **I** LCBD – Palearctic **J** LCBD change – Palearctic **K** LCBD – Sino Oriental **L** LCBD change – Sino Oriental. Values were standardised per biogeographic domain for better comparisons amongst them.

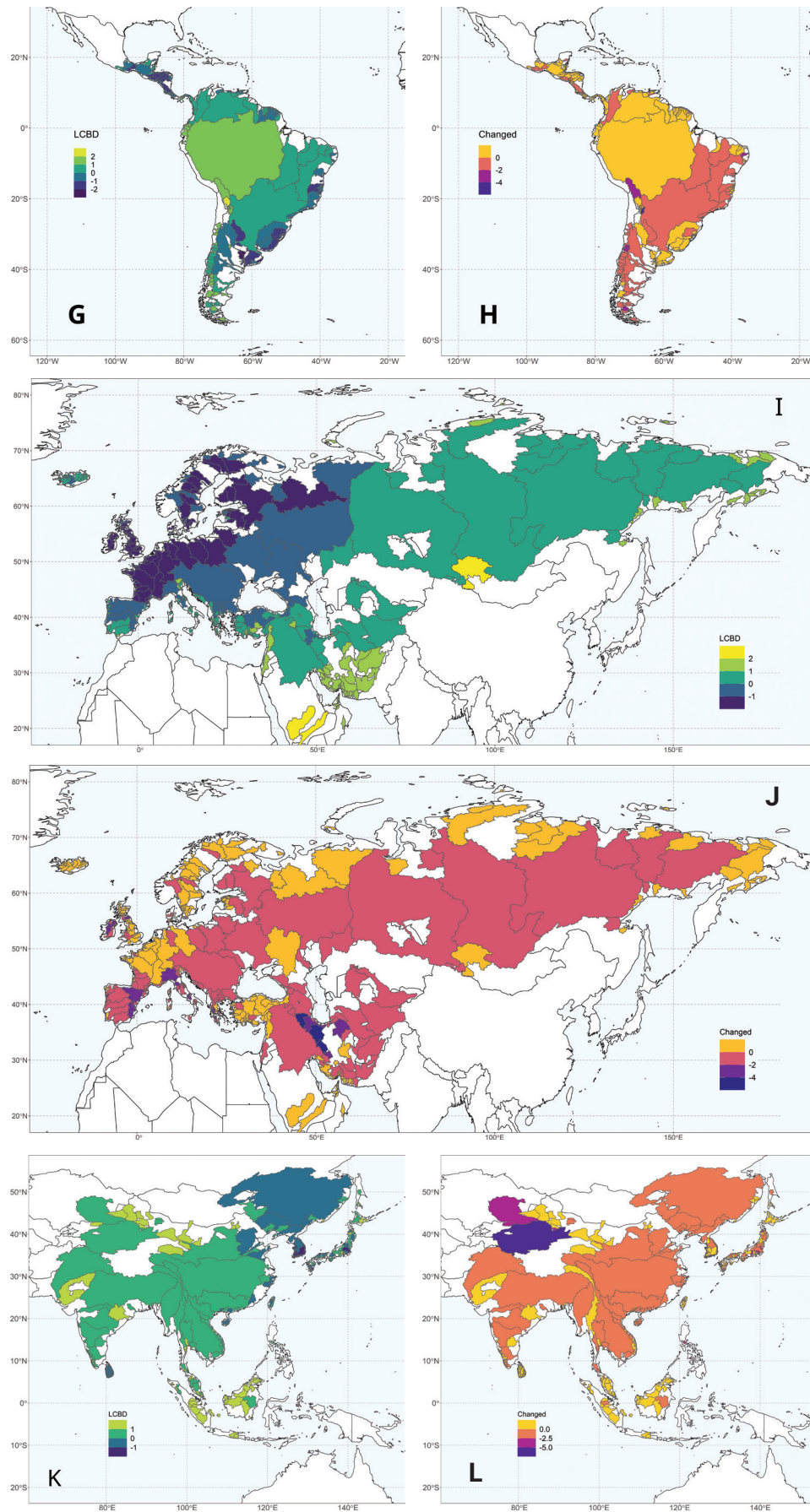


Figure 1. Continued.

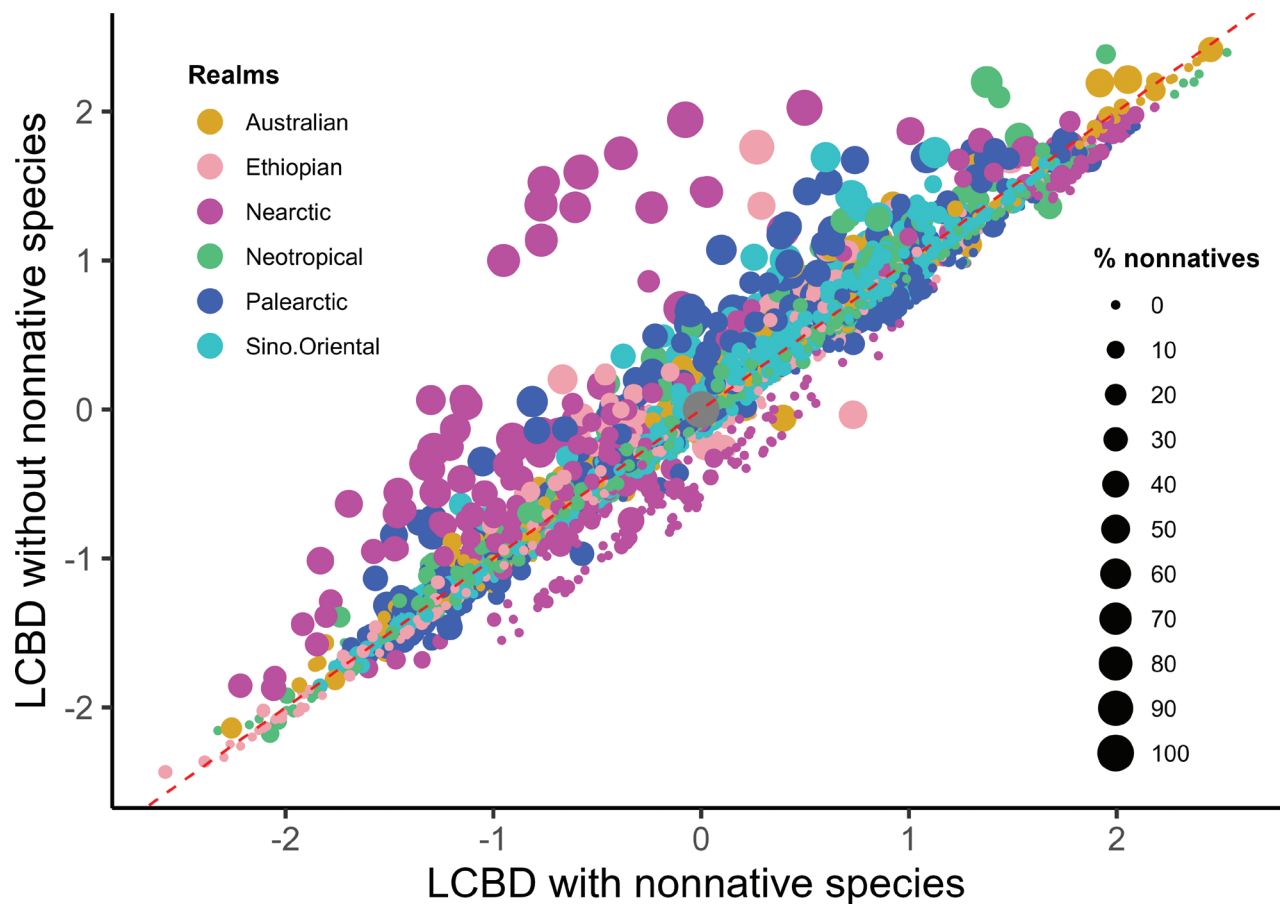


Figure 2. Relationship between standardised values of local contribution to beta-diversity (LCBD) with and without non-native species in each biogeographic domain. The size of the symbols indicates the proportion of non-native species and the colours indicate the domain. The red dashed line indicates the expected line of no change in LCBD. Graphs for each domain are available in Suppl. material 1.

Watershed species richness demonstrated weak associations with watershed contributions to beta-diversity (LCBD) (Fig. 4). The watersheds with the highest LCBD for each domain (those with unique conditions and species composition) rarely demonstrated high species richness. Even so, the watersheds with the highest species richness had LCBD values above average for Australian, Neotropical and Sino-Oriental domains (Fig. 4). The Suppl. material 1 summarises the features of the most important watershed in each biogeographical domain, including their nature (endorheic or exorheic) and overviews of species composition: IUCN status and endemism of species.

Although much less common worldwide (161 watersheds out of 2,760), endorheic watersheds had significantly higher LCBD (Fig. 4) in all domains (Welch two sample t-tests; $P < 0.001$), except Neotropical ($P = 0.775$) (Fig. 4). Detailing the features of the most important watersheds in each domain, it is clear that they included a unique species composition, with endemic and threatened species and most of them are endorheic (Suppl. material 1).

In general, SCDB values increased with occupancy (Fig. 5). SCDB was significantly higher for non-threatened species (Welch two sample t-tests; $P < 0.001$), except for Australian (Welch two sample t-tests; $P = 0.193$; see Fig. 5). Domain summaries are available in Suppl. material 1.

Native species always had the top five highest absolute SCDB values in all domains (Suppl. material 1), but non-natives had significantly higher mean SCDB

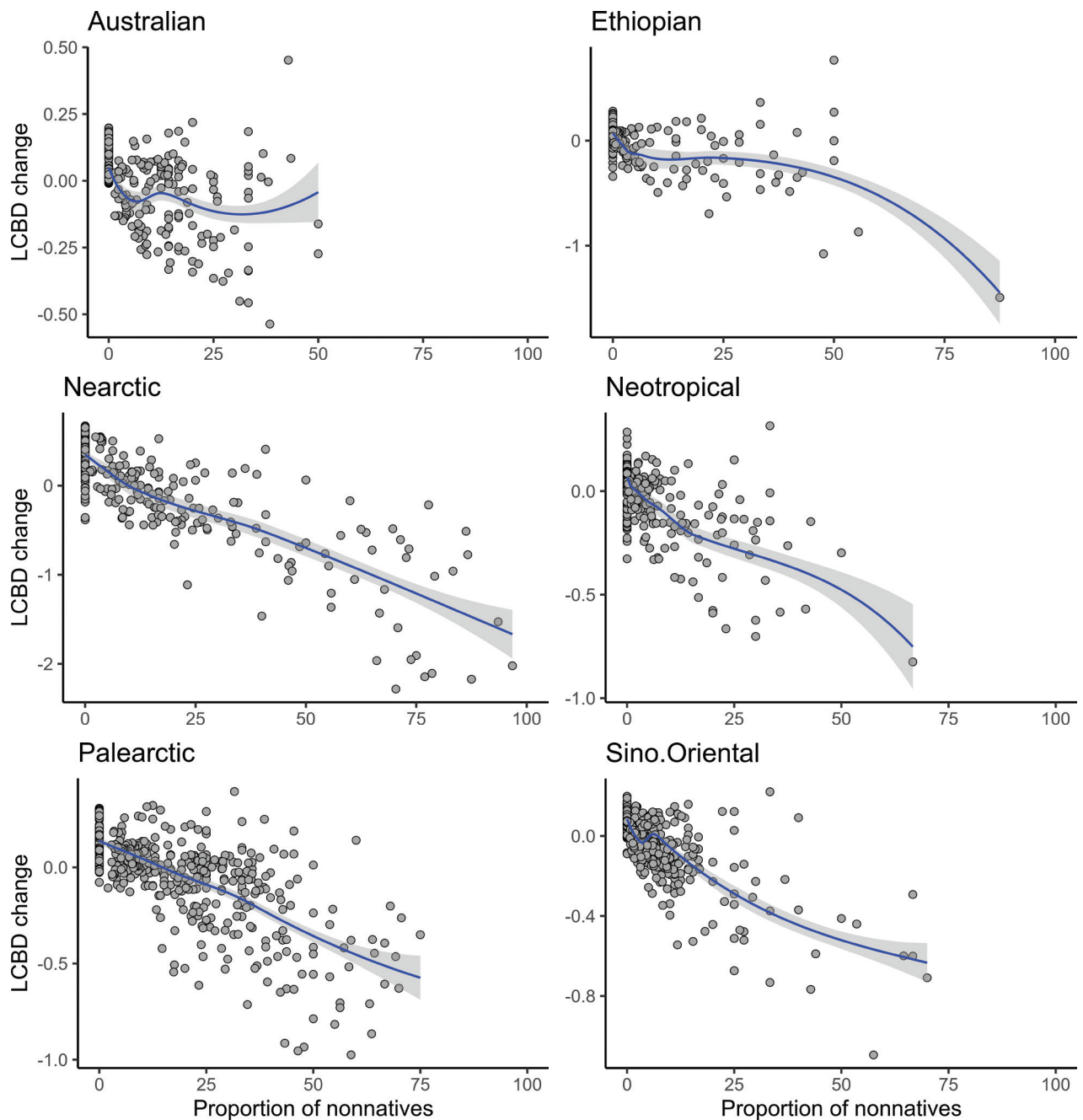


Figure 3. Changes in standardised values of local contribution to beta-diversity (LCBD change) considering values calculated with minus without non-native species against the proportion of non-native species (percentages) from each biogeographic domain. Curves indicate the best fit (grey areas are the standard errors) according to a local polynomial regression fitting method. R-squared were the following for each domain: Australian (28.4%); Ethiopian (40.0%); Nearctic (71.5%); Neotropical (47.6%); Palearctic (54.3%); Sino.Oriental (54.1%).

in Ethiopian and Neotropical domains (Welch two sample t-tests; $P = 0.008$ and $P = 0.04$, respectively, Fig. 5). The eastern mosquito fish *Gambusia holbrooki* (Girard, 1859) was the non-native species with the highest SCBD in three domains (8th in Australian, 6th in Ethiopian and 17th in Palearctic; native from Nearctic). The other non-native species with highest values in each domain were: the guppy fish *Poecilia reticulata* (Peters, 1859) (35th in Nearctic, native from Neotropical), the European trout *Salmo trutta* (Linnaeus, 1758) (7th in Neotropical, native from Palearctic) and the Mozambique tilapia *Oreochromis mossambicus* (Peters, 1852) (29th in Sino-Oriental, native from the Ethiopian).

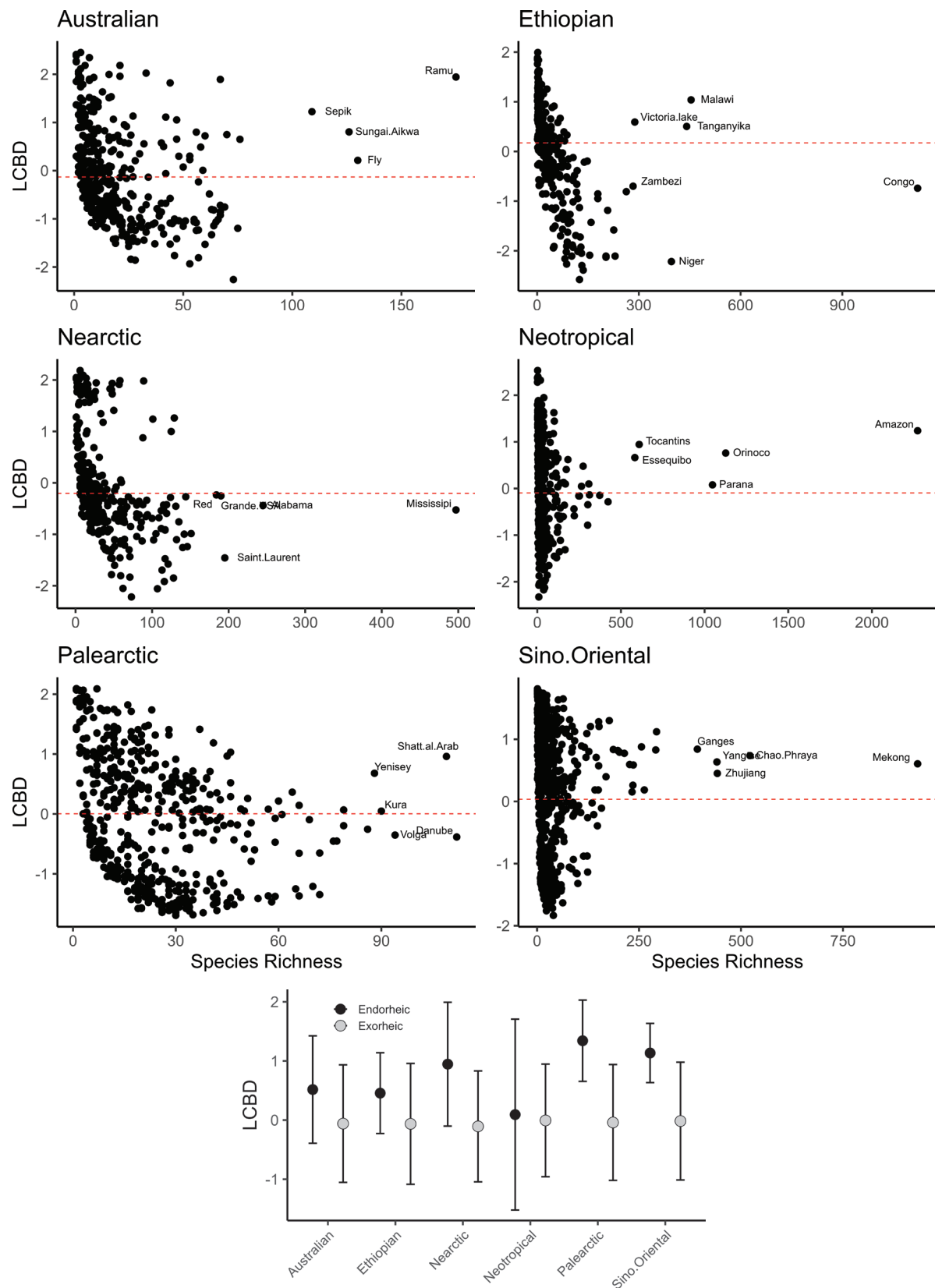


Figure 4. Relationship between standardised values of the local contribution of beta-diversity (LCBD) and species richness in the watersheds for each freshwater biogeographic domain (first six graphs). The horizontal red dashed line indicates the median value, so watersheds above it are the most important for species composition according to LCBD. Watersheds with the highest species richness were identified. Lower graph: means and standard deviations of the standardised LCBD for endorheic (black) and exorheic (grey) watersheds in the different biogeographic domains.

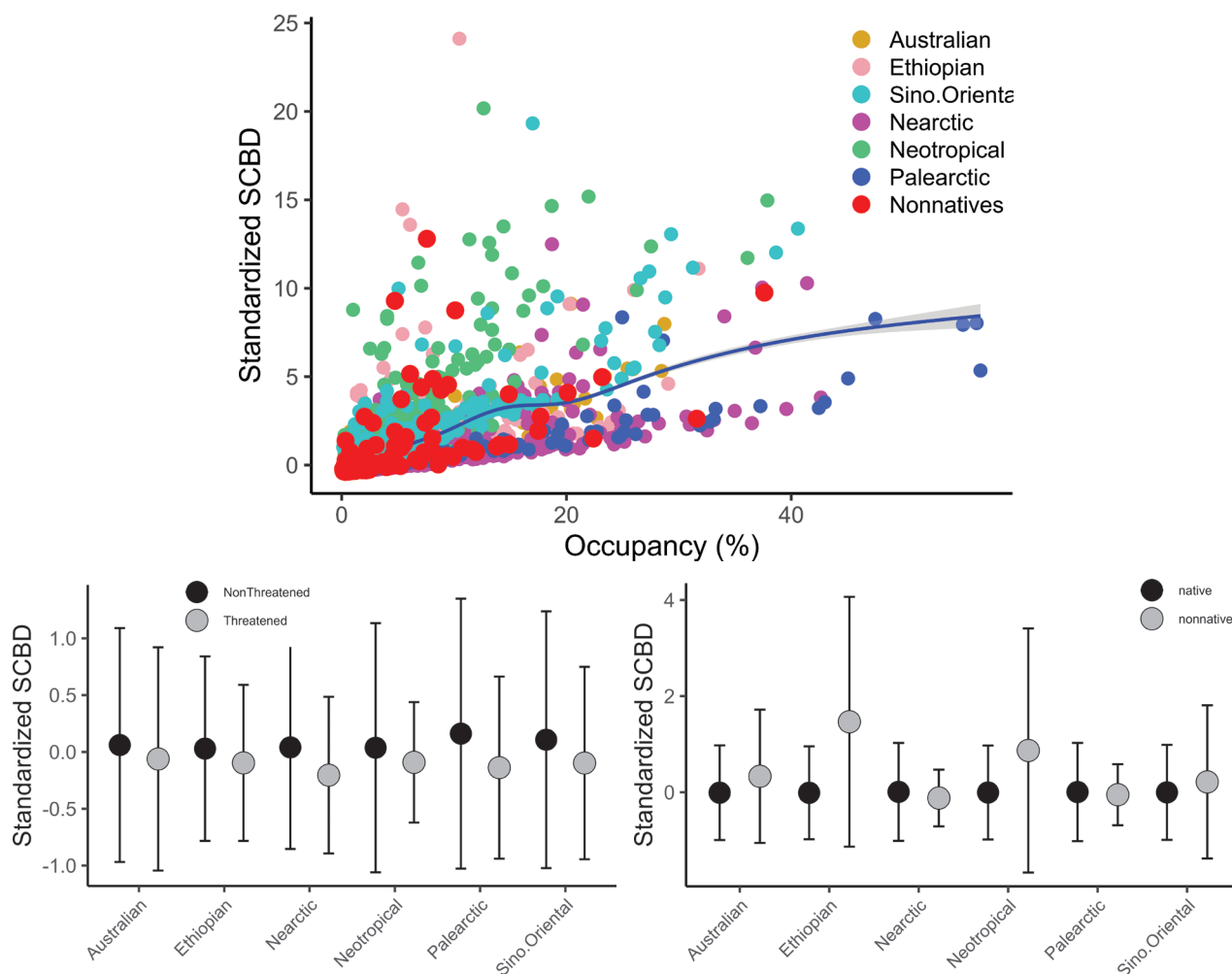


Figure 5. Upper middle: relationship between standardised values of species contribution to beta-diversity (SCBD) and relative species occupancy, estimated as the percentage of watersheds in which the species occur for biogeographic domains to which it belongs (R -squared to polynomial regressions = 51.6%). Values were standardised for each domain separately. Colours indicate standardised SCBD values for species in different biogeographic domains, red dots highlight the non-native species in biogeographic domains and blue lines indicate the best-fit curve (grey areas are the standard errors) using the local polynomial regression fitting method. Relationships for each biogeographic domain are available in the Suppl. material 1. Lower left: Means and standard deviations of standardised values of species contribution to beta-diversity (SCBD) between non-threatened (black) and threatened species (grey) *sensu* IUCN threat status in all biogeographic domains. Lower right: means and standard deviations of standardised values of species contribution to beta-diversity (SCBD) between native (black) and non-native species (grey) in all biogeographic domains.

Some patterns emerged amongst the top five species with the highest SCBD in each of these six domains: the majority of species were Euryaline, even though most species from the database live in freshwater or only tolerate estuaries (16 out of 30 species, 53.3%); a high prevalence of demersal or benthopelagic species (27 out of 30, 90.0%) and species with some migrating behaviour (18 out of 30, 60.0%); and five species (16.7%) having some reported IUCN vulnerability (three Endangered and two Near Threatened, see Suppl. material 1).

Discussion

Introductions by non-native species have fundamentally altered the global biogeography of freshwater fishes. This study demonstrates marked taxonomic and geographic differences in contributions to fish beta-diversity patterns of biogeographic

domains. Our results have important implications for national and international conservation initiatives that seek to preserve the uniqueness of the world's fish fauna.

We demonstrated a highly variable effect of non-native species on global-scale biogeographic patterns of freshwater fish. Despite this variability, in general, we found with increasing non-native species dominance comes greater reduction in LCBD and this effect occurred mainly in the domains with known human impacts (Leroy et al. 2023). This result is supported by continued evidence for fish faunal homogenisation being promoted by cosmopolitan non-native species replacing endemic native species over time (McKinney and Lockwood 1999). Indeed, evaluating the effect of non-natives at a global scale reveals the ability of invasions to reduce beta-diversity (Leprieur et al. 2008; Toussaint et al. 2016; Liu et al. 2017; Leroy et al. 2023). By highlighting the deterioration of beta-diversity by species invasions, our approach adds another piece to solve 'The Biodiversity Conservation Paradox' puzzle, in which Vellend (2017) argues that the number of species is not a good indicator for conservation priority without considering species origin and compositional variation, amongst other facets of biodiversity. We add our voices to the growing chorus that compositional uniqueness should be considered together with species richness in biodiversity assessments of conservation priorities.

It was clear that the Ethiopian, Nearctic, Palearctic and Sino Oriental are the domains in which the beta diversities of watersheds were mostly changed due to non-native species. Even so, we raised concerns in the Neotropical domain, given the watersheds with higher decreases in beta-diversity due to invasions were also those with higher importance for beta-diversity (see Fig. 2) in the most important domain for global fish biodiversity. The most important watersheds for such domains were those that have a great connection to coastal areas, in which marine freshwater-tolerant species (or vice versa) inhabit (see also Kong et al. 2017). This fact may even explain the watershed that increased LCBD due to the introduction of new species into the community from coastal and estuarine systems. The inclusion of freshwater non-native species may have influenced the taxonomic and even functional dissimilarity compared to other watersheds (Milardi et al. 2019). This rationale can also explain the fact that the Australian domain had watersheds that both increased (particularly those with low LCBD) and decreased (particularly those with high LCBD) beta-diversity due to non-native species, thus not resulting in a clear decrease pattern of LCBD with non-natives.

LCBD has been used as an important indicator of ecosystem uniqueness considering species composition (Bórquez et al. 2023). Our study reveals such important facets for global freshwater conservation: the watersheds that mostly contribute to beta-diversity in their domains are those with unique conditions that deserve conservation efforts. Although many context-dependent features may explain the uniqueness of watersheds, we could find some patterns: the most unique watersheds were not necessarily those with high species richness, they usually had high endemism, harbour species with IUCN threat status and, amongst them, endorheic watersheds deserve careful attention.

Endorheic watersheds are expected to be more unique and, at the same time, more susceptible to global changes due to their higher physical isolation and consequently high rates of species replacement and endemism (Levêque et al. 2008; Leprieur et al. 2011; Albert et al. 2020). Even though there were no significant differences in mean LCBD values for the Neotropical domain, the watershed with the highest LCBD values in this domain is a nice example: isolated and located

on the central plateau of the Andes (Bolivia-Argentina-Chile), ‘Salina de Uyuni’ is a unique place with low richness and high endemism of species that live under extreme salt and climatic conditions typical of the mountains. Although we did not find references to support the uniqueness of environmental conditions of this region, our results suggest that it should be better investigated and described in ecological studies. Indeed, only four genera of fish exist above 3,000 m in the Andean plateau, one of them recently described (Lacoste et al. 2020). Even so, mountain sites are currently threatened by the introduction of Salmonidae species (Vila et al. 2007; Aigo et al. 2008). Another extreme example can be observed in the endorheic watershed with the highest LCBD in the Nearctic: ‘Bolson de Sandia’ in Mexico. Five species from this watershed are now considered extinct or extinct in the wild according to the IUCN. Even without considering extinct species in our analyses, this watershed still had a high LCBD, harbouring a unique endemic species: the speckled flounder flatfish *Paralichthys woolmani* Jordan & Williams (1897). The second most important watershed in the Nearctic was also an endorheic watershed in Mexico: ‘Bolson de Cuatro Ciénegas’, with all six fish species either endangered or critically endangered by IUCN. Finally, the top three watersheds with the highest LCBD in Palearctic were all endorheic and composed mainly of endemic species, including the IUCN-vulnerable *Salmo abanticus* Tortonese (1954).

The species-rich watersheds in biogeographic domains did not have the highest LCBD values, but some had above-average values. The high LCBD and species-rich watersheds are also endorheic in the Ethiopian domain: the ancestral lakes Victoria, Tanganyika and Malawi, all with a high level of cichlid endemism (Lévêque et al. 2008). These lakes have seen the introduction of translocated species that now hybridise and compete with native species, causing some populations to decline or disappear altogether (Cucherousset and Olden 2011). Other species-rich watersheds had also above-average LCBD values in other domains, such as the Amazon in Neotropical, Mekong in Sino Oriental, Ramu in Australian and Shatt al Arab in Palearctic. Such watersheds are also amongst the largest in their domains, with a well-documented high proportion of endemic species, very important for socio-economic activities and also recently impacted by species introductions (Fu et al. 2003; Jellyman et al. 2015; Albert et al. 2020).

The contribution of watersheds to freshwater beta-diversity is just one of many important metrics to consider with respect to prioritising conservation action. For the Ethiopian domain, the two watersheds with the highest LCBD were characterised by endangered and endemic species, but the watershed with the highest species richness had a below-average LCBD: the Congo River watershed. The Congo River watershed has a relatively low percentage of non-natives and is less impacted than other watersheds of similar richness (Su et al. 2021). Thus, the reasons for its low uniqueness should be related to multiple palaeo-connectivity and eco-evolutionary mechanisms (see also Carvajal-Leprieux et al. 2011). Undoubtedly, this river should always be a priority for freshwater fish conservation in Ethiopian domains, considering other reasons (biodiversity, ecological and economic relevance, large size etc.) apart from its compositional uniqueness.

Another watershed that is highly biologically diverse, but not unique considering LCBD is the Danube watershed in the Palearctic. This river has a long history of anthropogenic pressure and is a conservation priority due to pollution, land use, urbanisation, alteration of the hydrological regime and the introduction of species; which has resulted in the disappearance of many native species (Bănăduc et al. 2020). In the

Nearctic domain, the most iconic watershed has a similar situation: the Mississippi River watershed, considered the greatest refuge for freshwater fish and with a high degree of endemism in the domain (Dias et al. 2014), but with below-average LCBD probably due to the high number of non-natives ($n = 66$), the second-most invaded watershed after the Colorado River (see Suppl. material 1). Considering the high number of native species ($n = 431$), the proportion of non-natives in the Mississippi River may not be high, but the impacts of non-natives are usually disproportional and due to certain species (Britton 2022). Indeed, the invasion of one well-suited fish can disrupt most communities (e.g. Pelicice and Agostinho 2009) and impacts are even more disastrous in sites with high endemism (Daga et al. 2016).

More than focusing on watersheds, we also shed light on the most important species contributing to beta-diversity in each domain. As expected, SCBD generally increases with regional occupancy (Pozzobom et al. 2020), but, noticeably, the vast majority of species have lower than 50% occupancy. The most important species were almost exclusively native in origin and inhabited the bottom of the water column, mostly with euryhaline habitats and with some kind of migratory behaviour. The biology of such species explains their higher SCBD, given they may vary more than freshwater-only non-migratory fish amongst watersheds. Relatedly, saline tolerance is key in explaining fish composition and diversity in freshwater-marine transition zones (Whitfield 2015).

The loss of species with the largest contributions to beta-diversity is, thus, most likely to promote biotic homogenisation (Olden 2006). Such species could be the focus of regional conservation efforts to ensure longer-term persistence. We found little evidence that IUCN threatened species have higher SCBD values compared to unlisted species. This result is somewhat expected given such species may likely be rare, with low occupancy in their domain and, thus, contribute little to global beta-diversity. We reinforce that this result may never be interpreted as a reason not to focus on their conservation. Promoting their conservation may increase their occupancy and, consequently, their importance to beta-diversity. Even so, considering only the top five species with the highest SCBD values in each domain ($n = 30$), five of them already have an IUCN status of threat and we urge for their conservation. In the Nearctic, the two native trout species rainbow trout *Oncorhynchus mykiss* Walbaum (1792) and brook trout *Salvelinus fontinalis* Mitchill (1814) are amongst them, although they can be considered non-native species in some basins of North America and globally. Conservation of native trout in USA has become a matter of intense debate given their historical link to human culture (e.g. Williams et al. 2015); our study reinforces the relevance of this discussion. Related, the near-threatened Atlantic salmon *Salmo salar* (Linnaeus, 1758) is ranked as one of the most important species in the Palearctic to beta-diversity and is also one of the best studied and culturally valuable fish species in the Northern Hemisphere. Here, we only reinforce its importance to the Palearctic beta-diversity conservation. In the Australian domain, the IUCN near-threatened southern pygmy perch *Nannoperca australis* (Günther, 1861) also deserves careful attention. This species is one of the small non-commercial fish that receive less attention to conservation and is now threatened mainly due to barriers to riverine movement (Todd et al. 2017). Finally, the endangered Japanese eel *Anguilla japonica* (Temminck & Schlegel, 1846) is another well-known species given its cultural significance and is threatened by multiple factors, such as non-controlled fisheries, habitat degradation and climatic changes (Yadav et al. 2020).

Another interesting view on the most important species considering their SCBD is the diversity of orders. Amongst the 30 most important species for the different domains, there were 13 different orders, which reinforces the context-dependence of freshwater fish diversity and origin (see van der Sleen and Albert 2021). As expected given their high diversity worldwide, Cypriniformes had the highest number of species listed as the most important. However, Salmoniformes was the order with the second highest number of important species, although it is only the third most diverse order in the Palearctic and the fourth most diverse in the Nearctic, where there are more species with the highest SCBD (van der Sleen and Albert 2021).

Here, we interpret high SCBD values as a proxy of the relative importance of species to beta-diversity (*sensu* Legendre and De Cáceres 2013). For native species, it is an informative metric to conservation efforts. On the other hand, the SCBD values of non-natives indicate their importance in changing domain beta-diversity, thus can be interpreted as an important metric to management efforts. Considering the non-natives with the highest SCBD values in each domain, they coincide with the species listed as the most frequently introduced species worldwide (see Suppl. material 1, fig. 3 in Muñoz-Mas et al. 2023), reinforcing that introductions disrupt global patterns of beta-diversity.

Final remarks

Distributions of non-native species are closely linked with human activities (Bernery et al. 2022) and their impacts are related to the characteristics of the receiving ecosystem as well as their ability to colonise new environments (Hui et al. 2023). Our study reinforces this already well-known pattern of decreasing beta-diversity mainly in the most impacted Nearctic and Palearctic domains (Leroy et al. 2023). Here, we scaled down and advocated that watersheds in this now-called PAGNEA were the ones that mostly changed their relative importance given non-native species. However, we went further and provided a map of priority watersheds, their overall features and a rank of species that should be considered for conservation efforts that praise the uniqueness of aquatic ecosystems. We also provided insights into how watersheds and species are more or less important to the biogeographic domain beta-diversity. We hope that, together with other biodiversity facets, our study contributes to a better understanding of the biogeographical patterns of freshwater fish and effective conservation planning.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Data availability

The organised data used for all analyses are available as Supporting Information and were obtained from freely available global databases.

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Supplementary material 1

Supporting information

Authors: Lorraine L. Cavalcante, Thiago V. T. Occhi, Julian D. Olden, Andre A. Padial

Data type: zip

Explanation note: 1) LCBD-Cavalcante_et_al-Available.csv 2) SCBD-Cavalcante_et_al-Available.csv 3) Occurrence_Data.csv 4) Relationship between standardised values of local contribution to beta-diversity (LCBD) with and without non-native species in each biogeographic domain 5) Relationship between species contribution to beta-diversity (SCBD) and relative species occupancy, estimated as the percentage of watersheds in which the species occur for the different biogeographic domains 6) Means and standard deviations of standardised values of species contribution to beta-diversity (SCBD) for all IUCN threat categories for each domain separately and for all domains together 7) Watersheds (according to Tedesco et al. 2017) with the highest beta-diversity (LCBD, all with similar values after five decimal numbers) in each biogeographic domain and their species composition.

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Research Article

The challenge of attaining conservation outcomes in a complex system: Agency personnel's and academic researchers' perspectives on the wicked problem of the exotic pet trade

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Abstract

The exotic pet trade is a wicked problem involving economic, social, political, ethical, and environmental dimensions, which cannot be resolved using conventional management strategies that are informed by restricted expertise. In 2023, we surveyed 26 government agency personnel and 57 academic researchers in the United States who focus on the risks of the exotic pet trade to ascertain how experts characterize the wicked problem of the exotic pet trade and their support for conventional versus collaborative management of the trade. Both academic and agency respondents framed the ecological risks associated with the exotic pet trade similarly, expressing greatest concern about species invasions and pathogen transmission to native species. Respondents exhibited low levels of trust in stakeholders in the exotic pet trade, considering it likely that all stakeholders (except the commercial industry) would fail to comply with pet trade regulations. Agency respondents tended to agree that current regulations have been effective in mitigating invasion risks while academic respondents disagreed that current regulations adequately mitigate the invasion and disease risks of the pet trade or overexploitation of species. Agency respondents were more likely to agree that regulations are enforceable. All respondents were supportive of additional federal regulations of the exotic pet trade. Our findings are consistent with the argument that wicked problems are perpetuated because managers and scientists default to conventional cause-effect problem statements and top-down management approaches that focus on management structure and execution. Transitioning from top-down regulatory approaches to collaborative decision making, in which agencies, scientists, and exotic pet trade stakeholders work together to resolve the wicked problem of the exotic pet trade, would build trust and allow for flexible, adaptive management of the trade.

Key words: Collaborative management, disease risk, invasion risk, management, policy, regulation, structured decision-making



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Introduction

Current conservation conflicts are typically wicked problems, which cannot be resolved using conventional management actions (Game et al. 2014; Mason et al. 2018). In their seminal paper, Rittel and Webber (1973) coined the term 'wicked problem' when they argued that policy problems are not mathematical problems

that can be efficiently resolved using scientific methods based on Newtonian mechanistic physics (which assumes that the universe is predictable, controllable, and law-abiding). Rather, conservation conflicts are complex, multifaceted issues with no clear solutions due to their interconnectedness, ambiguity, and diverse stakeholder involvement (Adams et al. 2019; Cosens et al. 2020). According to Rittel and Webber (1973), scientists and agencies fail to attain desirable, socially acceptable outcomes because they adopt an engineering approach in which scientific knowledge and models are applied to problems without first critically defining and locating problems. Scientists and agencies thus fail to distinguish between observed and desired conditions, assess the true causes of conflicts, or acknowledge that actions within complex socio-ecological systems may generate more severe problems (Rittel and Webber 1973; Game et al. 2014; Mason et al. 2018). The result is that the public and stakeholders lose trust in scientists and managers, who do not account for diverse and competing interests and world views, unintended and irreversible consequences of policy actions, and inequities (Rittel and Webber 1973; Game et al. 2014; Mason et al. 2018).

The exotic pet trade is indeed a wicked problem. Trade in exotic pets is a key driver of global environmental change (Robinson et al. 2015; Auliya et al. 2016b), contributing to invasion and disease risks, overexploitation of species, extinction of imperiled species, ecological disruptions, and biodiversity loss (Brown 2006; Hulme 2015). However, the exotic pet trade also involves social, economic, political, and ethical dimensions (Sinclair et al. 2021), intersecting with issues of animal welfare, public health risks (e.g., zoonotic diseases), economic well-being, cultural practices, competing stakeholder interests, and power inequities (Smith et al. 2012; Bush et al. 2014; Lockwood et al. 2019; Marra 2019). In defining the problem of the exotic pet trade, scientists and managers must account for ecosystem impacts, the welfare of traded animals (Pasmans et al. 2017; Warwick et al. 2018), legal frameworks (Pratt et al. 2024), social, psychological, and economic drivers of the pet trade (Hausmann et al. 2023; Pienaar and Sturgeon 2024), and different spatial scales at which impacts of the trade are manifest (Sinclair et al. 2021). Unfortunately, growing social and political conflicts pertaining to the exotic pet trade clearly demonstrate that conventional management strategies have failed to resolve the problems posed by the trade (Episcopio-Sturgeon and Pienaar 2019). We focus on the wicked problem of the exotic pet trade in the United States of America (US). The US contains the largest volume and most diverse array of exotic pets globally (Stringham et al. 2021) but inconsistent and incomplete regulations pertaining to the exotic pet trade have failed to mitigate the risks posed by the trade (Burgos-Rodríguez & Burgiel 2020; Pratt et al. 2024).

When considering the exotic pet trade, researchers have largely analyzed the ecological consequences of introducing non-native species into new environments (Brown 2006; Auliya et al. 2016a; Lockwood et al. 2019; Gippet and Bertelsmeier 2021), and the welfare of traded animals, specifically transportation, housing, and the psychological and physical well-being of exotic pets (Baker et al. 2013; Warwick et al. 2016; Whitehead 2016; Toland et al. 2020). Government agencies and NGOs, on the other hand, have typically focused on regulations to control and manage the trade, aiming to curb illegal trafficking and regulate ownership (Baker et al. 2013; Pratt et al. 2024). Agencies and NGOs have also focused on outreach efforts designed to educate the public about the ethical and ecological implications of the exotic pet trade and to reduce demand for and release of exotic

pets. However, these efforts have been undermined by unintended consequences (Levin et al. 2012), resistance by stakeholders with vested interests or conflicting world views (Waddock and Lozano 2013), changing societal values, technological advancements that make current efforts outdated, and increased online commerce (Bammer 2005; Oppenheimer 2011; Bertuol-Garcia et al. 2018). Management of the exotic pet trade has been further complicated by lack of comprehensive data on the number and species of exotic pets in the trade (Rhyne et al. 2012; Sinclair et al. 2021), limited agency resources and personnel to monitor and enforce written policies (Reeve 2006; Fonseca et al. 2021), jurisdictional barriers to interagency management of the exotic pet trade, and inconsistent regulations pertaining to the exotic pet trade (Pratt et al. 2024).

Despite clear evidence that the exotic pet trade is a wicked problem, managers and scientists may be resistant to pivoting from conventional “best practice” management approaches that are designed to address single objectives in simple environments to developing strategies that address complexity (Game et al. 2014; Mason et al. 2018). “Best practice” actions that treat the exotic pet trade as a conventional cause-effect problem are far easier to implement because they replicate past, known practices and are suited to current institutional structures (Game et al. 2014). Conventional management involves top-down decision making, which is informed by restricted expertise, and fails to predict the potential impacts of management decisions on complex, dynamic environments (Game et al. 2014; McEachran et al. 2024; Cook et al. 2025). Although agencies claim to engage in adaptive management, management actions are typically focused on feedback controls because this reduces cognitive effort and resources needed for planning by allowing managers to apply simple mental models and rules of thumb (Game et al. 2014; McEachran et al. 2024). Rather than investing in understanding complex systems, managers focus on management structure and execution (Game et al. 2014), which may result in a ‘Type III error’ (finding a good solution to the wrong problem; Mitroff and Silvers 2010).

To help inform efforts to improve the management of the exotic pet trade in the US, we conducted an initial, exploratory investigation of how experts characterize the wicked problem of the exotic pet trade and their support for conventional versus collaborative management of the trade. We focused on invasion scientists who work for state and federal government agencies and academic institutions in the US because these individuals comprise the experts who typically inform policy and management efforts. Although the US Fish and Wildlife Service (USFWS) is the primary federal agency that enforces laws related to the exotic pet trade, states retain most regulatory authority over the trade (Pratt et al. 2024). State agencies use a mix of prohibited lists of species that may not be legally imported or owned (commonly referred to in the wildlife trade literature as ‘blacklists’) and lists of approved species for import and ownership (commonly referred to as ‘whitelists’; Hulme 2015; Bowen 2021) to regulate the pet trade within their state (Pratt et al. 2024). We examined agency personnel and academic researchers’ 1) perceptions of the risks and benefits posed by the exotic pet trade, 2) knowledge of current exotic pet regulations, 3) perceptions of the effectiveness of current regulations in mitigating risks, 4) opinions on the constraints to effective regulation of the trade, and 5) support for additional management of the exotic pet trade. We further examined whether experts believed that state agencies’ risk perceptions pertaining to the exotic pet trade match their personal risk perceptions.

Methods

Questionnaire design

We designed an online questionnaire to be administered to individuals specializing in invasion science and working for universities or state and federal wildlife or agricultural agencies. In addition to asking respondents their gender, age, and education level, we collected information about their professional experience, the state they work in, the agency or university they work for, and their job titles.

Perceived risks and benefits associated with the exotic pet trade

Before asking agency personnel about the risks that the exotic pet trade poses, we asked them to indicate the exotic pets (hereafter, species) over which their agency has regulatory authority ('birds', 'rodents', 'large carnivores', 'primates', 'other mammals', 'reptiles', 'amphibians', 'fish', 'mollusks', 'crustaceans', 'insects/arachnids'). Agency personnel who stated that their agency had no regulatory authority over exotic pets, or they did not know which exotic pets their agency regulated, were asked to identify which species they thought posed risks within their state. We asked academic researchers which species they study or consider risks within their state.

For each of the species that respondents selected, we asked how concerned they were that trade in these species as pets would result in 1) transmission of pathogens to humans, native animals, and agricultural industries, 2) species invasions, 3) unregulated harvest of wild populations of species, and 4) extinction of native species ('not at all concerned', 'slightly concerned', 'moderately concerned', 'concerned', or 'very concerned'). We also asked respondents to indicate their state agency's concern pertaining to trade in these species as exotic pets ('N/A; I do not know' response provided for respondents who were uncertain about their agency's regulatory authority over the exotic pet trade). Finally, all respondents indicated their personal and agency concern about whether exotic pet owners would voluntarily comply with exotic pet regulations.

As a further measure of risk perceptions, we asked respondents the likelihood that the commercial pet industry (large wholesale and retail enterprises that breed or sell exotic pets at high volumes, and may be part of a national chain of stores), the hobbyist industry (smaller home-based enterprises that breed or sell pets at low volumes, and specialize in specific taxa or species of pets), the exposition industry (enterprises that participate in events where pet breeders and sellers gather to exhibit and sell pets, e.g. Repticon, <https://repticon.com/>), and exotic pet owners (who do not breed, sell, or exhibit exotic pets) would comply with existing exotic pet regulations, or engage in illegal behavior pertaining to the trade and release of pets ('very unlikely', 'unlikely', 'neither likely nor unlikely', 'likely', 'very likely', 'I do not know/I do not have any experience with this group'). We also asked respondents how likely they thought it was that these different stakeholders in the pet trade would contribute to invasion risks, pathogen transmission risks, overexploitation of wild populations, and species extinctions in the US ('not at all likely' to 'extremely likely'). Respondents indicated what percentage of the animals traded by the commercial, hobbyist, and exposition industries they thought were healthy (i.e., free of pathogens). Finally, respondents stated how often they thought exotic pet owners deliberately released their pets into the wild ('never', 'rarely', 'sometimes', 'often').

We assessed respondents' perceptions of the benefits of the exotic pet trade by asking them whether they agreed that the exotic pet trade is beneficial to the economy, and the exotic pet trade helps conserve endangered species ('strongly disagree', 'disagree', 'neither agree nor disagree', 'agree', 'strongly agree').

Knowledge of exotic pet regulations

We assessed all respondents' knowledge of current exotic pet regulations in their state (I know 'all', 'some', or 'none' of the exotic pet regulations in my state). To measure agency respondents' knowledge of their agency's regulatory authority over the exotic pet trade, we asked if their agency had jurisdiction over the exotic pet trade ('yes, all exotic pets', 'yes, some exotic pets', 'maybe/I am not sure', and 'no'), as well as what type of regulation their agency uses ('whitelists', 'blacklists', 'both whitelists and blacklists', 'neither'). We further asked if respondents were familiar with proposed amendments to the federal Lacey Act ('yes', 'no', 'I do not know'). Recently, to promote increased enforcement of regulations for the exotic pet trade, the House of Representatives proposed HR 4521 (i.e., America COMPETES Act or Lacey Act Amendments; hereafter, Lacey Act Amendments). Three fundamental changes to the importation of species into the US and interstate trade of animals were proposed, namely: 1) the creation of a list of approved species that can be imported into the US, where any animal not listed is treated as an injurious species by default and banned from importation; 2) changes to the Lacey Act shipment clause to ban the interstate transport of species listed as injurious; and 3) conferral of new emergency powers that would provide the USFWS with the ability to prohibit the importation of injurious species for up to three years. If passed, the Lacey Act amendments would reduce inconsistencies in exotic pet trade regulations. Finally, we asked agency respondents to list other agencies (if any) with jurisdiction over the exotic pet trade in their state.

Perceptions of current exotic pet regulations

All respondents indicated how effective they thought exotic pet regulations in their state (as currently written) were in preventing invasion risks, disease risks, overexploitation of species, and species extinction ('not at all effective', 'slightly effective', 'moderately effective', 'effective', 'very effective', 'I do not know'). To obtain further insights, we asked respondents if, to the best of their knowledge, current regulations are being enforced, or can realistically be enforced in their state. Respondents also indicated whether they agreed that 1) jurisdictional barriers between agencies, 2) lack of agency funding and, 3) lack of trained agency staff prevents effective regulation of the pet trade, and that 4) agency staff have difficulty in identifying different species ('strongly disagree' to 'strongly agree'). As a final measure of respondents' perceptions of the effectiveness of regulations, we asked whether they agreed that current regulations adequately mitigate invasion risks, disease risks, overexploitation of species, and extinction risks, and that current regulations are enforceable ('strongly disagree' to 'strongly agree').

Support for altered regulation and management of the exotic pet trade

Respondents indicated whether they supported trade in native and non-native animals as pets within their state ('strongly oppose', 'somewhat oppose', 'neither oppose nor support', 'somewhat support', 'strongly support'). We assessed

respondents' support for proposed exotic pet regulations by asking them whether they support the Lacey Act Amendments. Respondents also stated whether they thought collaboration between their state agency and the commercial, hobbyist, and exposition industries (i.e., co-management of the exotic pet trade) would be 1) likely to occur ('not at all' to 'extremely' likely), and 2) effective in improving compliance with exotic pet regulations ('not at all' to 'very' effective). Finally, respondents indicated whether the risks associated with the exotic pet trade would be effectively mitigated by implementing 1) a three-day waiting period before a potential owner can collect a pet from the store, 2) allowing owners to return their animal to the pet store at no penalty to them, 3) tagging animals through skin or pit tags before they can be taken home so owners of released pets can be identified, and 4) adding a sales tax to exotic pet purchases to help fund agency efforts to regulate the pet trade.

Pretesting and ethics approval

We pretested this survey with four human dimensions, strategic communication, and invasion ecology experts. The Institutional Review Board at the University of Georgia reviewed the final survey and determined it was exempt (ID: PROJECT00006638).

Data collection

Data collection occurred from January to April 2023. We compiled a list of publicly available email addresses for adults (aged ≥ 18 years) who engaged in invasion science and worked for universities or state or federal wildlife or agricultural agencies across the US. We targeted these experts because they are most likely to inform or implement management actions, based on their understanding of 1) which species pose invasion risks within the state in which they reside, 2) what measures (e.g., trade regulations) are needed to mitigate these invasion risks, and 3) how state agencies have responded to invasion risks. We sent an initial email invitation to participate in our study, followed by ≤ 9 reminders (~ 2 /month) if participants had not completed the survey. Once participants completed the survey, they did not receive reminders. Individuals who elected not to participate in our study received no follow-up emails.

Data analysis

We performed all statistical analyses with SPSS version 28.0 and R version 4.0.4. We used Kruskal Wallis H tests to test for differences in 1) survey responses by agency personnel and academic researchers and 2) responses pertaining to personal versus agency risk perceptions.

Results

We received 26 agency personnel responses from 99 email-delivered surveys ($\sim 26\%$ response rate), with 20 responses from state agency representatives and six from federal agency representatives. We received 57 academic researcher responses from 146 email-delivered surveys ($\sim 39\%$ response rate).

Most respondents (62.7%) identified as male (Table 1). The median age category for respondents was 45–54 years, and the median education level was an advanced degree. Most academic respondents had ≥ 11 years of professional experience in invasion science, with job titles including associate professor, unit leader, and research biologist/ecologist. Most agency respondents had ≥ 11 years of professional experience, and had worked 3–5 years in their current position, with job titles including conservation biologist, division director, program coordinator, and research manager.

Perceived risks and benefits associated with the exotic pet trade

Agency respondents most frequently reported that small mammals (excluding rodents, 61.5%), reptiles (53.8%), amphibians (50.0%), birds (50.0%), and fish (50.0%) were the species over which their agency had regulatory authority (Suppl. material 1: table S1). Academic respondents most frequently reported that they researched or were concerned about risks posed by fish (63.2%) and mollusks (54.4%). Agency and academic respondents expressed similar levels of concern pertaining to species invasions, pathogen transmission to native species, agricultural industries, and humans, unregulated harvest, and extinction of wild populations owing to the exotic pet trade (Fig. 1; Suppl. material 1: tables S2–S7). However, agency and academic respondents differed in their perceptions of the state agency's concern about trade in crustaceans resulting in the extinction of wild species ($H(1)=3.888$, $p = 0.049$).

Table 1. Demographic characteristics and professional experience of survey respondents, January–April 2023 ($n = 83$).

	Agency ($n = 26$)		Academic ($n = 57$)		Total ($n = 83$)	
	Number	%	Number	%	Number	%
Gender						
Male	15	57.7	37	64.9	52	62.7
Female	10	38.5	20	35.1	30	36.1
Prefer not to say	1	3.8	0	0.0	1	1.2
Age						
25–34 years	1	3.8	4	7.0	5	6.0
35–44 years	9	34.6	17	29.8	26	31.3
45–54 years	11	42.3	16	28.1	27	32.5
55–64 years	5	19.2	14	24.6	19	22.9
≥ 65 years	0	0.0	6	10.5	6	7.2
Education						
Bachelor's degree	7	26.9	0	0.0	7	8.4
Advanced degree	19	73.1	57	100.0	76	91.6
Length of employment						
< 1 year	0	0.0	1	1.8	1	1.2
1, 2 years	0	0.0	0	0.0	0	0.0
3–5 years	2	7.7	4	7.0	6	7.2
6–10 years	6	23.1	10	17.5	16	19.3
≥ 11 years	18	69.2	42	73.7	60	72.3
Time in current position						
< 1 year	3	11.5	-	-	-	-
1, 2 years	4	15.4	-	-	-	-
3–5 years	7	26.9	-	-	-	-
6–10 years	6	23.1	-	-	-	-
≥ 11 years	6	23.1	-	-	-	-

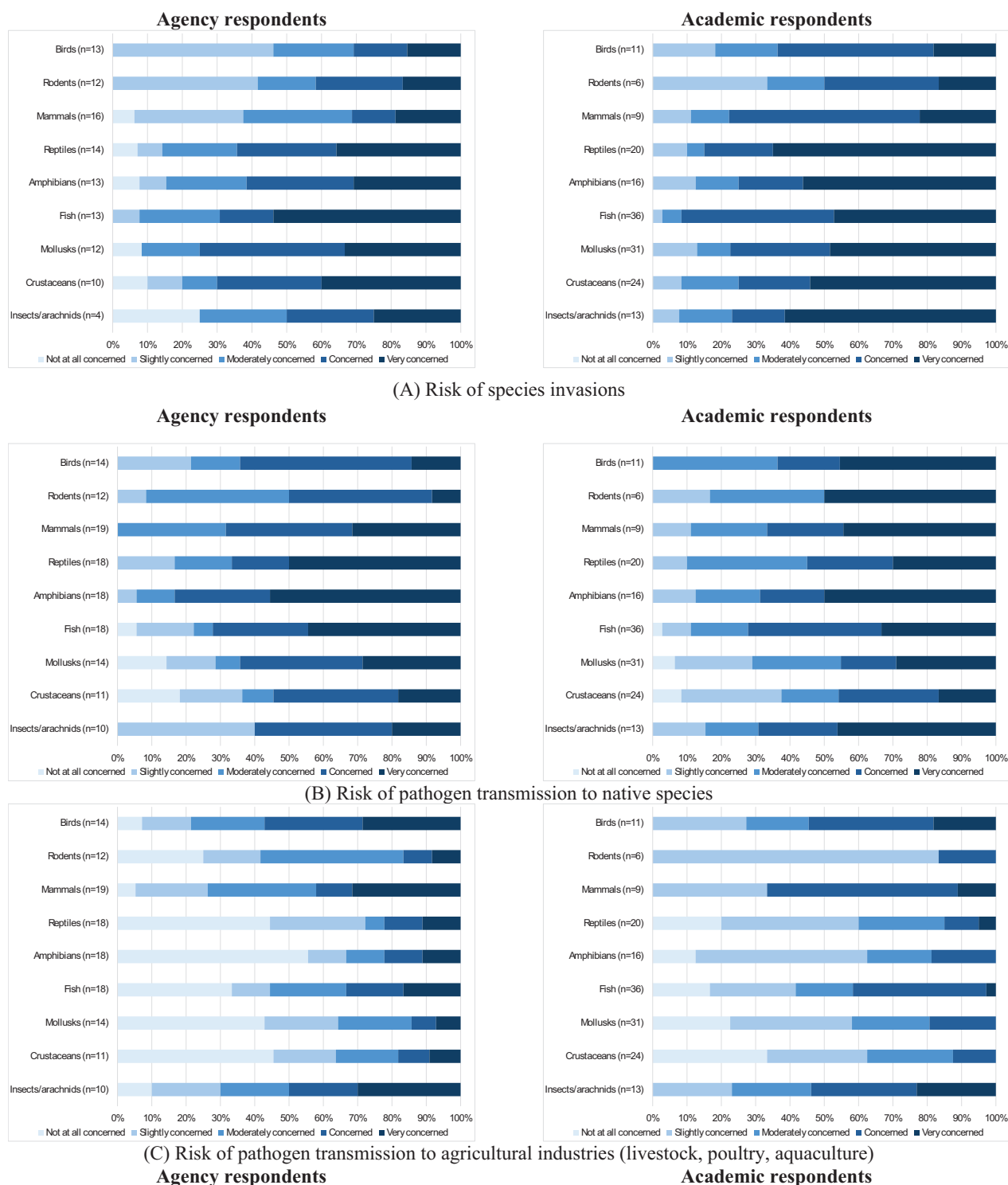


Figure 1. Respondents' risk sensitivity to threats posed by the exotic pet trade. Respondents answered the question: "How concerned are you about the possibility that trade in the following animals (as pets) will result in **A** species invasions **B** pathogen transmission to native species **C** pathogen transmission to agricultural industries (livestock, poultry, aquaculture), and **D** pathogen transmission to humans in your state **E** unregulated harvest of wild species, and **F** extinction of wild species within the US?" 'Mammals' exclude rodents, primates, and large carnivores.

Agency respondents' personal concern about the risks posed by the exotic pet trade was similar to their perceptions of how concerned agencies were about these risks. Academic respondents' personal concern about pathogen transmission risks and the risks of unregulated harvest of wild populations

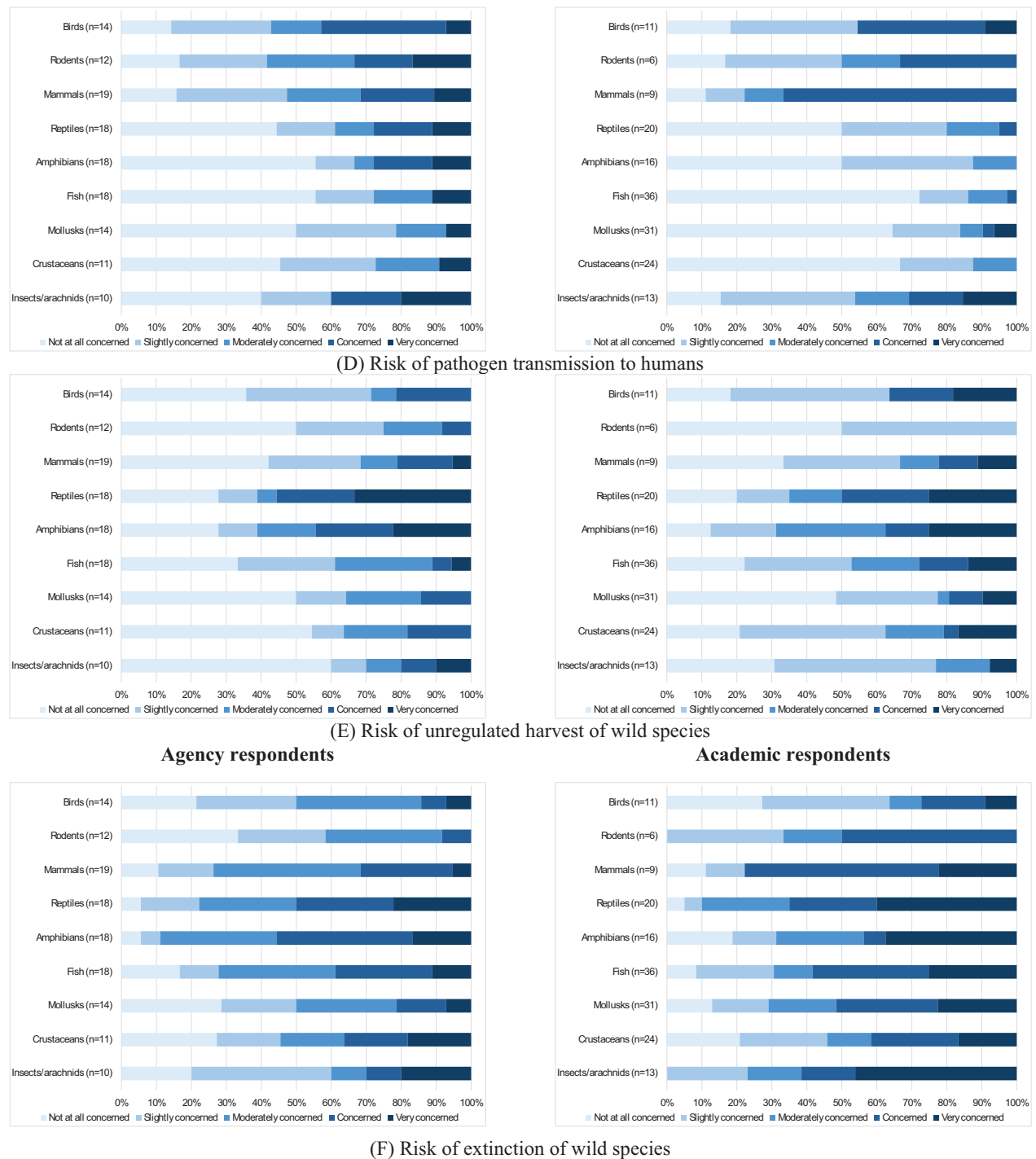


Figure 1. Continued.

was also similar to perceived agency concern about these risks. However, academic respondents stated that, in their opinion, they were more concerned than agencies were that trade in pet amphibians and reptiles (median='very concerned') would result in species invasions (amphibians: median='moderately concerned'; $H(1)=5.847$, $p = 0.016$; reptiles: median='concerned'; $H(1)=5.168$, $p = 0.023$; Fig. 2). Similarly, academic respondents stated that they were more concerned (median='concerned') than agencies (median='moderately concerned') that trade in pet reptiles would result in extinction of wild populations ($H(1)=4.969$, $p = 0.026$).

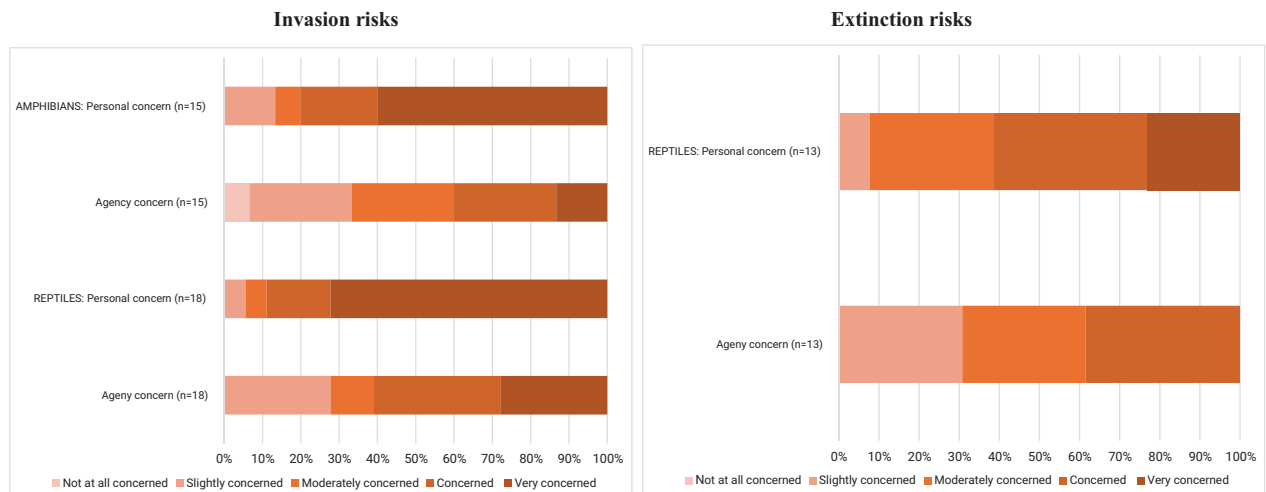


Figure 2. Academic respondents' personal concern about the invasion and extinction risks associated with the exotic pet trade, and their perceptions of state agencies' concern about these risks. Respondents who stated that they did not know how concerned the state agency was about these risks are excluded from the figure.

On average, agency and academic respondents were concerned about exotic pet owners voluntarily complying with pet trade regulations (Suppl. material 1: table S8). Agency respondents indicated that their concern about pet owners complying with regulations was similar to the state agency's perceived concern about compliance. In contrast, academic respondents argued that their concern about pet owners complying with regulations exceeded agency concern ($H(1)=8.588$, $p = 0.003$).

Academic and agency respondents expressed similar opinions about which pet trade stakeholders would comply with current regulations and illegally trade and release exotic pets. They considered that hobbyist breeders and sellers, the exposition industry, and exotic pet owners were unlikely to comply with regulations and were likely to illegally trade and release exotic pets, whereas the commercial industry would be likely to comply with regulations and unlikely to engage in illegal behaviors (Suppl. material 1: fig. S1, tables S9, S10). Agency and academic respondents also expressed similar opinions about the probability that these four stakeholder groups would contribute to invasion risks (mean responses for agency respondents ranged from 6.2–7.0, mean responses for academic respondents ranged from 6.9–8.0 where 'not at all likely'=0 and 'extremely likely'=10; Suppl. material 1: fig. S2), pathogen risks (agency respondents: $6.5 \leq \text{mean} \leq 7.0$, academic respondents: $6.1 \leq \text{mean} \leq 7.1$; Suppl. material 1: fig. S3) and species extinctions in the US (agency respondents: $4.1 \leq \text{mean} \leq 4.6$, academic respondents: $4.3 \leq \text{mean} \leq 4.9$; Suppl. material 1: fig. S4, Table S11). However, academic respondents thought the hobbyist industry was more likely to contribute to the overexploitation of wildlife in the US (6.3 ± 2.7) than agency respondents (4.8 ± 2.6 ; $t = -2.454$, $p = 0.009$; $H(1)=5.873$, $p = 0.015$; Suppl. material 1: fig. S5). On average, respondents thought that 57.8% of animals traded by the commercial industry were healthy, 55.0% of animals traded by the hobbyist industry were healthy, and 50.2% of animals traded by the exposition industry were healthy (Suppl. material 1: table S12). Most respondents (90.4%) thought exotic pet owners deliberately release their exotic pets into the wild at least sometimes (Suppl. material 1: table S13). Most respondents also disagreed that the exotic pet trade benefits the economy (54.2%) or helps to conserve endangered species (88.0%; Suppl. material 1: table S14).

Knowledge of exotic pet regulations

In total, 10 agency respondents (38.5%) and 7 academic respondents (12.3%) were aware of all exotic pet regulations in their state (42.3% of agency respondents and 68.4% of academic respondents were aware of some regulations). The majority of respondents (65.4% of agency respondents, 66.7% of academic respondents) were unaware of the Lacey Act amendments prior to the survey. Regarding regulatory approaches, 33.3% of agency respondents indicated that their agency uses lists of prohibited species, while 4.8% reported using lists of species that may be legally owned and traded. Additionally, 33.3% of agency respondents stated that their agency employs both types of lists (prohibited and authorized species), 23.8% reported using neither type of list, and 4.8% were uncertain or unaware of their agency's regulatory approach. Finally, 81.0% of agency respondents reported shared jurisdiction over the exotic pet trade, primarily between the state agricultural and wildlife agencies.

Perceptions of current exotic pet regulations

On average, agency respondents considered current exotic pet regulations in their state to be moderately effective in preventing disease risks, invasion risks, overexploitation of species, and extinction, whereas academic respondents considered regulations to be slightly effective (Suppl. material 1: fig. S6, table S15). Agency respondents were more likely to agree that current regulations adequately mitigate invasion risks (median='somewhat agree') than academic respondents (median='somewhat disagree'; $H(1)=4.884$, $p = 0.027$; Fig. 3, Suppl. material 1: table S16). Agency respondents were also more likely to agree that current regulations adequately mitigate species overexploitation (median='neither agree nor disagree') than academic respondents (median='somewhat disagree'; $H(1)=6.552$, $p = 0.010$).

Respondents most frequently stated that current regulations are enforced sometimes (61.9% of agency respondents, 45.7% of academic respondents; Suppl. material 1: table S17). Only seven agency respondents (33.3%) and 11 academic respondents (23.9%) thought regulations could realistically be

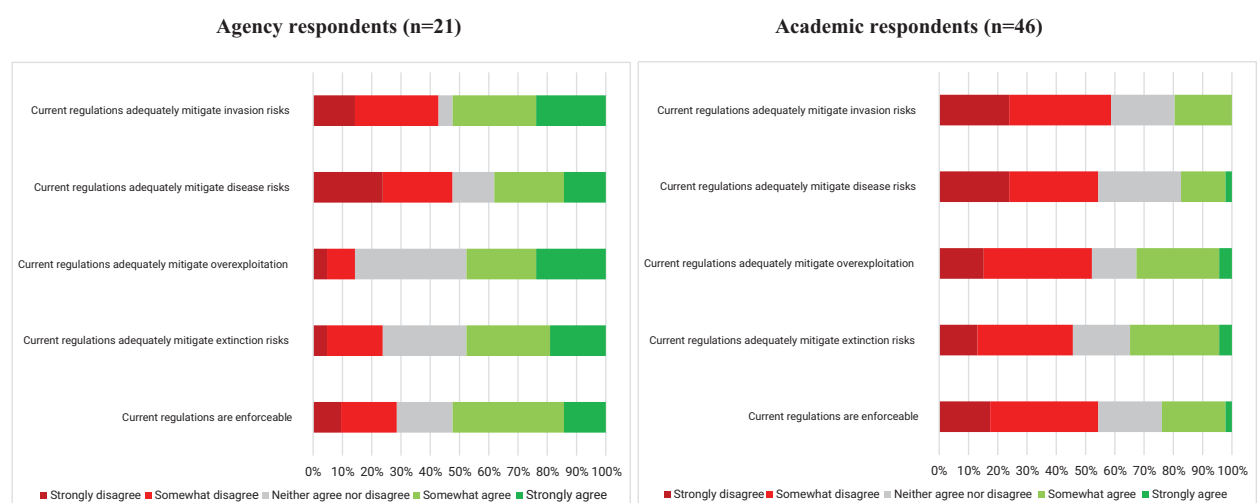


Figure 3. Respondents' agreement with statements about exotic pet regulations in their state.

enforced in their state. Agency personnel were more likely to agree that current regulations are enforceable (median='somewhat agree') than academic researchers (median='somewhat disagree'; $H(1)=5.468$, $p = 0.019$). Respondents most strongly agreed that lack of agency funding prevents effective regulation of the exotic pet trade (Fig. 4, Suppl. material 1: table S18). On average, respondents neither agreed nor disagreed that agency staff have difficulty in identifying different species in the pet trade.

Support for altered regulation and management of the exotic pet trade

On average, both agency and academic respondents somewhat opposed the sale of non-native animals as pets in the US (Suppl. material 1: table S19). However, agency respondents were more opposed to the sale of native species as pets in the US than academic respondents ($H(1)=12.951$, $p < 0.001$). Agency and academic respondents did not differ in their support for proposed Lacey Act amendments (Table 2). Most respondents supported the creation of a whitelist of approved species that can be imported, changing the Lacey Act shipment clause to ban the interstate transport of species listed as injurious, and new emergency powers allowing the USFWS to prohibit the importation of injurious species for up to three years.

Academic respondents considered it more likely that state wildlife agencies and the commercial industry would collaborate to manage the exotic pet trade (5.2 ± 2.6 ; range: 0 to 10) than agency respondents (3.6 ± 3.3 ; range 0 to 10; $H(1)=5.474$, $p = 0.019$). Agency and academic respondents did not differ in their assessment of the likelihood that the hobbyist (agency respondents: 3.6 ± 2.8 ; academic respondents: 3.9 ± 2.1) and exposition industries (agency respondents: 3.2 ± 2.8 ; academic respondents: 3.6 ± 2.2) would collaborate with the state wildlife agency to manage the pet trade.

On average, respondents indicated that collaboration between the state agency and the hobbyist and exposition industries would be moderately effective at enforcing pet trade regulations (Fig. 5, Suppl. material 1: table S20). However, academic respondents believed that co-management of the pet trade with the

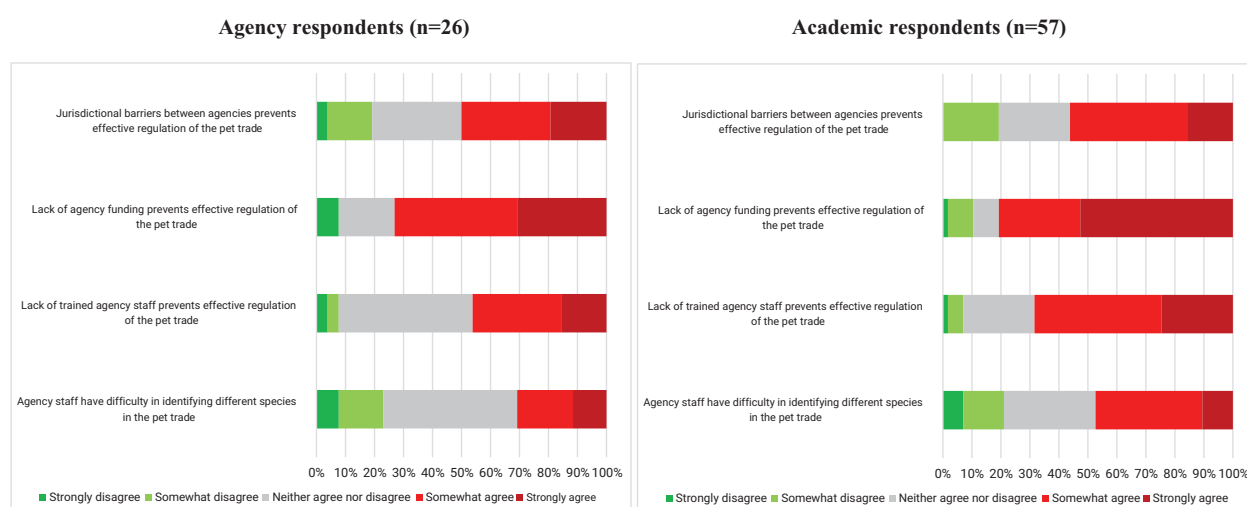


Figure 4. Respondents' opinions on barriers to effective regulation of the exotic pet trade. Note: We found no statistical difference in the distribution of responses across agency and academic respondents.

Table 2. Respondents' support for proposed Lacey Act amendments, January-April 2023 (n = 83).

	Median	N	Strongly oppose	Somewhat oppose	Neither support nor oppose	Somewhat support	Strongly support
Agency respondents:							
Create a list of approved species that can be imported, where any animal not listed is treated as an injurious species by default and banned from importation into the US.	Somewhat support	26	0 (0.0%)	4 (15.4%)	4 (15.4%)	10 (38.5%)	8 (30.8%)
Change the Lacey Act shipment clause to ban the interstate transport of species listed as injurious.	Somewhat support	26	0 (0.0%)	0 (0.0%)	2 (7.7%)	13 (50.0%)	11 (42.3%)
Establish new emergency powers that would provide the US Fish and Wildlife Service (USFWS) with the ability to prohibit the importation of injurious species for up to three years.	Strongly support	26	0 (0.0%)	0 (0.0%)	3 (11.5%)	8 (30.8%)	15 (57.7%)
Academic respondents:							
Create a list of approved species that can be imported, where any animal not listed is treated as an injurious species by default and banned from importation into the US.	Somewhat support	57	2 (3.5%)	1 (1.8%)	10 (17.5%)	19 (33.3%)	25 (43.9%)
Change the Lacey Act shipment clause to ban the interstate transport of species listed as injurious.	Strongly support	57	2 (3.5%)	0 (0.0%)	3 (5.3%)	17 (29.8%)	35 (61.4%)
Establish new emergency powers that would provide the US Fish and Wildlife Service (USFWS) with the ability to prohibit the importation of injurious species for up to three years.	Strongly support	57	1 (1.8%)	0 (0.0%)	5 (8.8%)	17 (29.8%)	34 (59.7%)

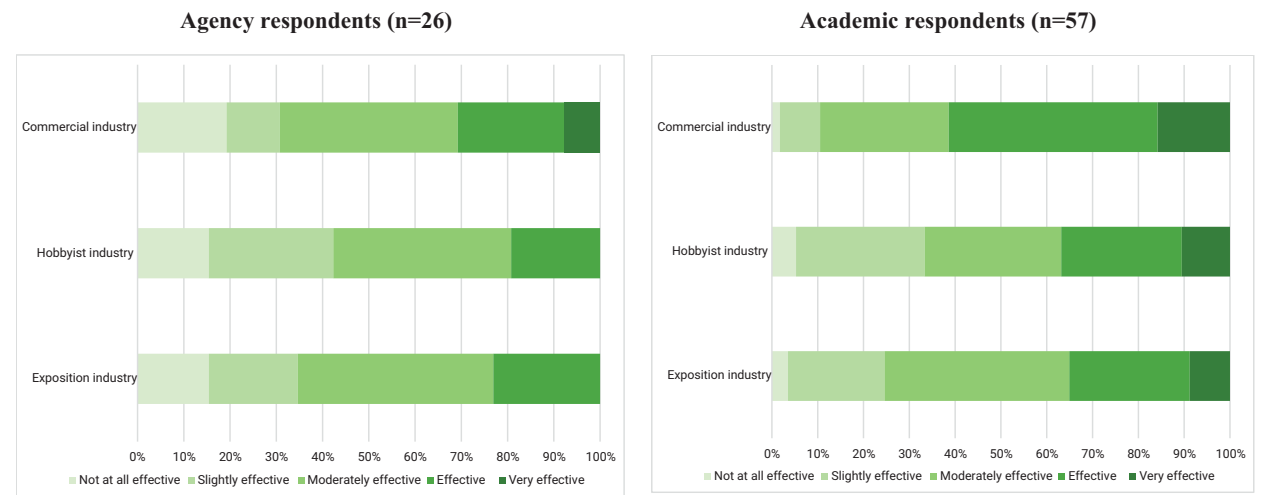


Figure 5. Distribution of responses to the question, “How effective would a collaboration between your state wildlife agency and the following groups be at helping enforce pet trade regulations?”

commercial industry would be more effective at enforcing pet trade regulations (median=‘effective’) than agency respondents (median=‘moderately effective’; $H(1)=7.908$, $p = 0.005$). Academic respondents also believed that adding a sales tax to exotic pet purchases to help fund agencies responsible for regulating the pet industry would be more effective at reducing the risks associated with the exotic pet trade (median = ‘effective’) than agency respondents (median = 2.5 where ‘slightly effective’ = 2 and ‘moderately effective’ = 3; $H(1) = 15.453$, $p < 0.001$; Fig. 6, Suppl. material 1: table S21). Agency and academic respondents did not differ in their agreement on the effectiveness of a three-day waiting period for pet purchases (median=‘slightly effective’), skin or pit tags for exotic pets (median=‘moderately effective’), and allowing owners to return unwanted pets (median for agency respondents=‘moderately effective’, median for academic respondents=‘effective’) in mitigating the risks of the exotic pet trade.

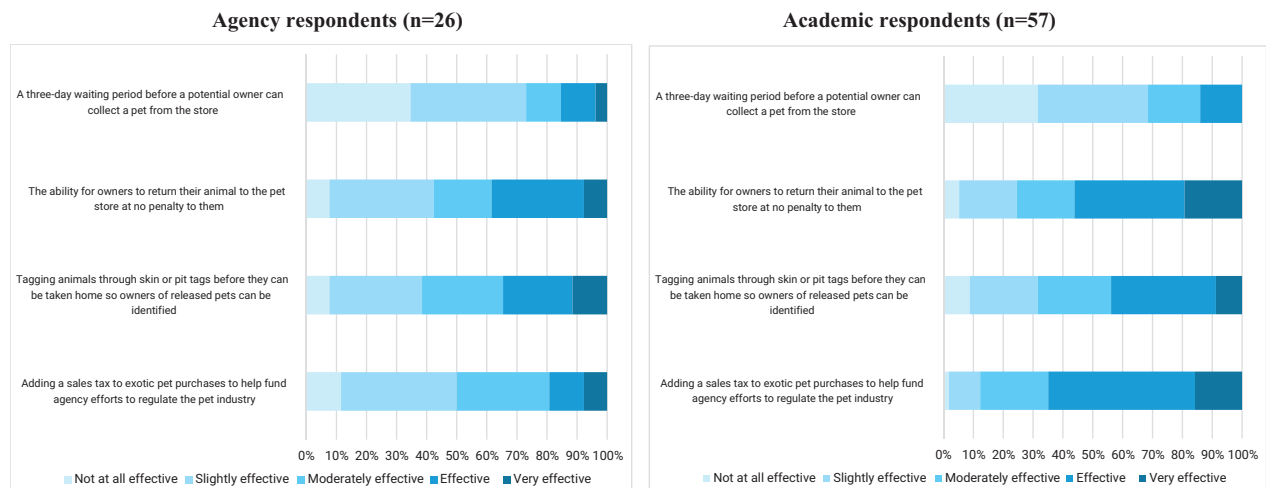


Figure 6. Distribution of responses to the question, “Assuming that the following management actions could be implemented in your state, please indicate how effective they would be at reducing the risks associated with the exotic pet trade”.

Discussion

The exotic pet trade presents a wicked problem, where researchers play a pivotal role in understanding the potential risks of the trade and government agencies play a pivotal role in managing these risks. We recognize that not all research scientists focus on applied research that is intended to inform management and decision-making. We specifically targeted managers and academics who focus on invasion science within the larger fields of wildlife, fisheries, and agriculture because most authority for managing the exotic pet trade lies with wildlife, fisheries, and natural resource agencies (Pratt et al. 2024), whose personnel are likely to be trained in biological and veterinary sciences. As such, current and proposed management of the exotic pet trade in the US are largely informed by restricted expertise. Our findings are consistent with arguments that decision and governance impediments result in managers and scientists defaulting to conventional cause-effect problem statements and piecemeal management approaches, rather than collaborative solutions that would more effectively address the wicked problem of the exotic pet trade (Game et al. 2014; Mason et al. 2018; Cook et al. 2025).

There were logical inconsistencies in how invasion scientists at academic and government institutions in the US framed the wicked problem of the exotic pet trade, their evaluation of the effectiveness of current top-down management approaches, and their stated support for additional top-down regulation of the exotic pet trade. Agency and academic respondents often framed the ecological risks associated with the exotic pet trade similarly, disagreeing that the exotic pet trade helps to conserve endangered species, expressing greatest concern about species invasions and pathogen transmission to native species, and suggesting that over half of the animals in the exotic pet trade are not healthy. However, respondents were less concerned about pathogen transmission to humans and agricultural industries and unregulated harvest of species – which are important adverse consequences of the exotic pet trade. Agency respondents may have expressed less concern about zoonotic and livestock disease risks because existing agency mandates and directives undermine incentives for wildlife agencies to manage zoonotic and livestock pathogen risks, which are largely under the authority of agricultural and human health agencies (Cook et al. 2025). Both agency and academic respondents agreed that lack of agency funding prevents

effective regulation of the pet trade, which is consistent with arguments that limited agency resources and capacity hinder management (Cook et al. 2025). Agency respondents also acknowledged that their agencies only regulate a subset of the species in the exotic pet trade, which limits their authority to mitigate exotic pet trade risks (Pratt et al. 2024; Cook et al. 2025). Yet, agency respondents tended to agree that current regulations are enforceable and have been 'moderately effective' or 'effective' in mitigating risks posed by the pet trade. This was inconsistent with most respondents' beliefs that exotic pet owners sometimes or often release their exotic pets into the wild, and that all stakeholders (except the commercial industry) are unlikely to comply with pet trade regulations and are likely to engage in illegal behavior pertaining to the trade and release of pets. Academic respondents demonstrated fewer logical inconsistencies, disagreeing that current regulations adequately mitigate the risks posed by the pet trade. Despite concerns about the trade in, and release of, pets that pose invasion and pathogen risks, respondents expressed doubts about implementing measures that would provide exotic pet owners with time to reconsider the purchase of a pet, the ability to return unwanted pets (academic respondents considered this to be more effective than agency respondents), identifying which owners release pets through skin or pit tags, and increased agency funding through the implementation of a sales tax on exotic pets (academic respondents considered this to be more effective than agency respondents). Rather, most respondents supported additional federal regulations in the form of the Lacey Act amendments – possibly because federal regulations would address inconsistencies in state regulations and would provide agency respondents with clear rules pertaining to management of the exotic pet trade (Pratt et al. 2024). However, it is not clear how federal regulations would address limited agency resources and personnel to monitor and enforce written policies (Reeve 2006; Fonseca et al. 2021).

Strong arguments can be made that multi-sector, collaborative governance is necessary to attain improved management of the exotic pet trade (Mason et al. 2018). Transitioning to co-management approaches, in which exotic pet trade stakeholders collaborate with scientists and government agencies to design and implement interventions, may result in increased trust between agencies, scientists, and stakeholders in the pet trade, and improved management of the exotic pet trade (Mason et al. 2018; Episcopio-Sturgeon and Pienaar 2019). Strategies from decision science and negotiation theory can be used to transition to collaborative governance of the exotic pet trade (Cook et al. 2025), even when experts have differing views and there is uncertainty about the likely effectiveness of different policies. Structured decision-making (SDM) is a formal values-based decision analytic framework and process that integrates social and ecological data to identify 1) clearly defined decision problems, 2) different objectives that reflect what different groups care about most, 3) alternative management actions, 4) barriers to decision-making (e.g., incomplete knowledge, risk, uncertainty, resource limitations, limited authority), and 5) tradeoffs among objectives (Runge et al. 2020). Agencies (managers), experts (scientists), and stakeholders (exotic pet trade) could engage in participatory SDM to identify possible interventions to mitigate the ecological and disease risks of the exotic pet trade while also considering the socio-psychological values and economic priorities of the trade.

The SDM process ensures that decision-making related to complex, contentious issues incorporates science and values in a transparent process that is robust to uncertainty (Runge et al. 2020). SDM uses data and expert opinion as the basis for predicting the likelihood that each alternative policy will fulfill the various desired objectives. Our study shows that experts differ in their assessments of how well

different interventions would achieve various objectives for exotic pet regulation. Differences in expert opinions about outcomes of interventions are a challenge for policymaking related to wicked problems. SDM is a particularly helpful tool for navigating that challenge. SDM explicitly incorporates the uncertainty that arises due to differences in expert opinion, tracking it through complex chains of cause-effect relationships, from intervention to expected outcomes. The likelihood of resulting predictions is adjusted to account for that uncertainty, thus offering a robust basis for decision-making even under uncertainty.

Importantly, SDM can build trust between agencies, scientists, and stakeholders (Robinson et al. 2016). This is a critical consideration in addressing the wicked problem of the exotic pet trade. Respondents' current lack of trust in pet trade stakeholders was manifest in their opinion that state agencies, hobbyist breeders and sellers, and exposition industries are unlikely to collaborate to manage the exotic pet trade, and that such collaboration would be moderately effective in enforcing regulations. However, agency respondents thought it was moderately likely that state agencies and the commercial industry would collaborate to manage the trade, and academic respondents thought collaboration between state agencies and the commercial industry would be effective at enforcing regulations. Engaging agency and academic researchers in decision-making processes that rigorously explore collaborative management alternatives could foster productive dialogue and reduce political and social conflicts between the exotic pet trade, scientists, and managers (Stout et al. 1999; Harris et al. 2010; Stern and Coleman 2015; Episcopio-Sturgeon and Pienaar 2019). Involving the exotic pet industry in collaborative management could generate a sense of ownership and responsibility, thereby encouraging industry stakeholders to adhere to regulations, voluntarily adopt best practices to mitigate the risks of the trade, and participate in conservation efforts.

Objectives identified through a multi-sector, multi-disciplinary SDM process could be used to identify possible management actions or interventions. For example, interventions may include changes to exotic pet regulations (Pratt et al. 2024), redistribution of resources to improve the capacity of agencies to respond to the risks posed by the exotic pet trade, education and communication campaigns that promote behavior change by exotic pet owners, and expanded adoption networks for unwanted exotic pets (Episcopio-Sturgeon and Pienaar 2019). Social scientists would play an important role in the SDM process by eliciting exotic pet trade participants' feedback and suggestions on different management objectives, a critical step to building trust between the exotic pet trade, scientists, and decision-makers (Robinson et al. 2016). Expert elicitation, empirical data, statistical and simulation models, and surveys of the exotic pet trade can be used to predict the consequences of different management actions in terms of invasion and disease risks, economic impacts, and support by the exotic pet trade (Robinson et al. 2016). Finally, the relative costs and impacts of different management actions can be determined, allowing participants to clearly see how each intervention is likely to satisfy each objective, and have transparent discussions about which interventions are preferable, given constraints and tradeoffs.

SDM related to the exotic pet trade should include experts in invasion science, public health (Can et al. 2019; van Roon et al. 2019), animal health, welfare, and transport (de Vos et al. 2017; Sutherland et al. 2021), social sciences (Robinson et al. 2016), strategic communication, and law, in order to identify management objectives with the potential to minimize the risks of the exotic pet trade while also securing the economic and social benefits of the trade. Disease ecologists and public health experts

provide important scientific insights on pathogen transmission through the exotic pet trade and actions that would reduce the risk of pathogen transmission to native species, domesticated animals, and humans. Social scientists provide insights into human behavior, motivations, and decision-making processes (Bennett et al. 2017), and can assist in navigating transdisciplinary collaboration (Marchini et al. 2021). Specialists in strategic communication offer valuable insights into effective messaging and conflict resolution strategies (Gregg et al. 2022). Strategic communication ensures that decisions are communicated clearly, the rationale for decisions is understood, and buy-in is obtained from relevant parties, thereby fostering collaboration in the implementation of decisions and reducing misconceptions (Werder 2014). Involving legal experts in decision-making ensures that policies and regulations are legally sound and comply with existing laws and regulations (Schwarz 2008; McEl-downey et al. 2013), and reduces loopholes and opportunities for non-compliance.

Addressing the wicked problem of the exotic pet trade requires collaborative engagement between different scientific disciplines, government agencies, and key stakeholders in the exotic pet trade to identify management actions. This is an extremely challenging task that requires scientists and managers to pivot from current conventional scientific and management models to embrace uncertainty and complexity. We recognize that SDM requires time, effort, and active engagement by different government agencies, an array of different scientific disciplines, and the exotic pet trade. However, collaborative, multidisciplinary management can generate shared understanding between stakeholders that facilitate innovative, adaptive problem-solving, particularly in addressing wicked problems where complexity and uncertainty of management arise (Stout et al. 1999; Lauber et al. 2011). SDM has been used to address conflicts related to game species harvests (Robinson et al. 2016), wildlife disease management (McEachran et al. 2024), and species invasions (Pepin et al. 2022). Reframing management of the exotic pet trade with decision analysis can re-establish trust in scientists and managers by accounting for diverse and competing interests and world views, the consequences of policy actions, and power dynamics related to the exotic pet trade (Rittel and Webber 1973; Game et al. 2014; Mason et al. 2018).

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

This study involved human subjects research in the form of online surveys. Our study was reviewed and approved by the appropriate Human Research Ethics Committee. The Institutional Review Board at the University of Georgia reviewed the final survey and determined it was exempt (ID: PROJECT00006638).

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Author contributions

Pratt: Conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing - original draft. Lockwood: Methodology, visualization, writing - review and editing. King: Methodology, visualization, writing - review and editing. Pienaar: Conceptualization, investigation, methodology, supervision, visualization, writing - original draft, writing - review and editing.

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Data availability

The data underpinning the analysis reported in this paper are deposited at Zenodo, and are available at <https://doi.org/10.5281/zenodo.14802808>.

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Supplementary material 1

Additional results for reference by readers and the reviewers

Authors: Elizabeth N. Pratt, Julie L. Lockwood, Elizabeth G. King, Elizabeth F. Pienaar

Data type: docx

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Research Article

Food fight: *Gammarus tigrinus* demonstrate competitive advantage over native *G. duebeni*

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Abstract

Introductions of non-native species (NNS) are major drivers of biodiversity loss. Gammarids (Crustacea, Gammaroidea) have been particularly successful in establishing and spreading in their non-native range, especially in Europe. While their impacts are wide-ranging, interference competition with native species has received limited study to date. Here, we assessed the competitive abilities of the successful North American NNS *Gammarus tigrinus* relative to the European native *Gammarus duebeni*, over a chironomid larva as a single food resource. We staged four types of dyadic contest encounters, with individuals of the native or NNS added to the experimental arena containing the food resource, and inter- or intraspecific competitor individuals added upon the first individual taking possession of the resource, or after 20 minutes. *Gammarus tigrinus* were more likely to take hold of the bloodworm in the opening 20 minutes, and did so more quickly than *G. duebeni*. During this period, they were also less thigmotactic than the native, being more explorative and spending a smaller proportion of time in the outer zone of the arena. They exhibited more aggressive interactions and activity with increasing size and mass, whereas larger *G. duebeni* were shown to be less aggressive and less active. *Gammarus tigrinus* were found to be significantly less likely to lose possession to *G. duebeni* than they were to conspecifics, whereas *G. duebeni* were similarly likely to lose possession to *G. tigrinus* as to conspecifics. Overall, our findings indicate that the behaviour and competitive ability of *G. tigrinus* demonstrated here add to a list of traits that facilitate its invasion success. In addition, our method offers potential as an effective, standardisable means of assessing the competitive abilities of gammarid NNS. We encourage future studies to develop it further, incorporating alternative resources, such as habitat, and to assess the role of ecologically relevant abiotic stressors in determining contest outcomes.

Key words: Animal behaviour, Baltic Sea, contests, exploitative competition, impact assessment, interference competition, non-native species

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Introduction

Global translocations of non-native species (NNS) are major drivers of biodiversity loss, affecting ecosystem services, human health and welfare, food security, and economic costs (Pyšek et al. 2020, Cuthbert et al. 2021, IPBES 2023). Amphipod crustaceans of the family Gammaroidea are one group of particularly damaging NNS, which have been highly successful at displacing natives, especially in Europe (Grabowski et al. 2007, Cuthbert et al. 2020). Gammarids are significant drivers of disturbance through competition for food and habitat resources, as well as predation, with major implications for native biodiversity in recipient freshwater and brackish ecosystems (Conlan, 1994). One such example is the North American *Gammarus tigrinus*, which has been shown to have negative impacts on native amphipod assemblages in introduced ecosystems (Jänes et al. 2015, Reissalu et al. 2016), as well as socio-economic costs to fishermen through damaged fishing gear and injured catches (Pinkster et al. 1977). Native to the brackish waters of tidal estuaries along North America's Atlantic coast, the first European occurrence was in England in 1931, likely arriving in ship ballast water (Sexton and Cooper 1939). From here, it was introduced to Northern Ireland in the 1950s before spreading southwards, replacing the native *G. lacustris* and *G. duebeni celticus* as the dominant gammarid in Lough Conn's sublittoral zone (O'Grady and Holmes 1983). It arrived and established in mainland Europe when the English stock was deliberately introduced to the polluted Werra river in Germany in 1957, and subsequently when the Lough Neagh population was introduced to IJsselmeer in the Netherlands in 1964. Since then, its spread has led to cases of replacement or population decreases for the native *G. pulex*, *G. zaddachi* and *G. d. duebeni* (Nijssen and Stock 1966, Pinkster et al. 1992, Kazanavičiūtė et al. 2024). In 1975, it reached the German part of the Baltic Sea, and from there its coastal range expansion has continued (see Rewicz et al. 2019 and references therein).

The Baltic Sea is the world's largest brackish-water basin and a highly unique ecosystem, with a low number of native species, many of which are postglacial immigrants, and at least 132 non-native and cryptogenic species (Casties et al. 2016, Ojaveer et al. 2017). Here, the combination of few native species, environmental instability and high anthropogenic pressure means the ecosystem is deemed sensitive to biological invasions (Reissalu et al. 2016, Rewicz et al. 2019). Indeed, *G. tigrinus* is joined by another North American gammarid NNS in *Melita nitida*, as well as Lake Baikal's *Gmelinoides fasciatus*, and seven NNS from the Ponto-Caspian region: *Chaetogammarus warpachowskyi*, *Chelicorophium curvispinum*, *Dikerogammarus villosus*, *D. haemobaphes*, *Chaetogammarus ischnus* (formerly *Echinogammarus ischnus*: Copilaş-Ciocianu et al. 2023), *Spirogammarus major* (formerly *E. trichiatus*: Copilaş-Ciocianu et al. 2023), *Obesogammarus crassus*, and *Pontogammarus robustoides* (Rewicz et al. 2019). Of these, *G. tigrinus* is viewed as one of the most euryhaline (Grabowski et al. 2006). While various studies have looked at the environmental tolerance of the species (Casties et al. 2019, Paiva et al. 2020), its life history traits (Pinkster et al. 1977, Grabowski et al. 2007) and even its ability to facilitate the consumption of congeneric native species by predators (Kotta et al. 2010), there remains a gap in the literature with regards to interference and exploitative competition outcomes over food resources with native gammarids. More broadly, there has been little study of contests to understand interspecific interactions, and its role on niche partitioning, species coexistence and biodiversity (Paijmans and Wong 2017), with even less in an invasion ecology context (but see, for example, Zeng et al. 2019).

Heeding recent calls for more studies to help decipher the competitive mechanisms at play between NNS and native species (Damas-Moreira et al. 2020), we paired *G. tigrinus* with *G. duebeni*, which has been outnumbered or replaced by the NNS in Ireland, the Netherlands and in the Baltic Sea (e.g. Vistula Lagoon, Poland: Grabowski et al. 2006; Dassower See, Germany: Kazanavičiūtė et al. 2024). Staging a series of dyadic contest experiments over a finite food resource, we used an experimental setup that involved staging four types of contest pairings: *G. tigrinus* vs *G. tigrinus*, *G. duebeni* vs *G. duebeni*, *G. tigrinus* vs *G. duebeni* and *G. duebeni* vs *G. tigrinus*. We sought to investigate species behaviour and determine the role of species identity, as well as body size and mass (traditional proxies for fighting ability, termed “resource holding potential”: Arnott and Elwood 2009) in determining contest outcomes. Specifically, we assessed boldness, activity and competitive ability using eight key tests. Boldness was assessed by examining the latency to approach the resource (1) and thigmotactic behaviour (2). Next, we assessed activity via the number of line crosses (see Fig. 1) per time in the experimental arenas (3), and finally, we assessed competitive ability (4–8). Boldness, i.e. how individuals behave in potentially risky situations (Réale et al. 2007), has been suggested to be a determinant of whether individuals are likely to disperse or remain sedentary, or whether they are short or long-distance dispersers (Fraser et al. 2001). This, alongside activity, has been highlighted as a beneficial behavioural trait across multiple stages of the introduction process (Chapple and Wong 2016), and they have been positively correlated in a number of invasive NNS (Brodin and Drotz 2014, Lukas et al. 2021). With *G. duebeni* having no known non-native populations (Paiva et al. 2018, Cuthbert et al. 2020), we propose these traits will be less obvious for the native species. As a result, we hypothesised that: (1) expecting it to be bolder, the non-native *G. tigrinus* is more likely than the native *G. duebeni* to take hold of the food resource in the opening 20 minutes, and of the individuals from both species taking hold of the food resource during that period, *G. tigrinus* is faster to take possession of the resource; (2) for the period of time that the individual added first is alone in the arena, *G. tigrinus* is less thigmotactic, i.e. spends less time in the outer ring of the arena; and (3) *G. tigrinus* is more active than *G. duebeni*. In terms of their competitive ability over food resources, our hypotheses were informed by previous studies which have shown *G. tigrinus* to be

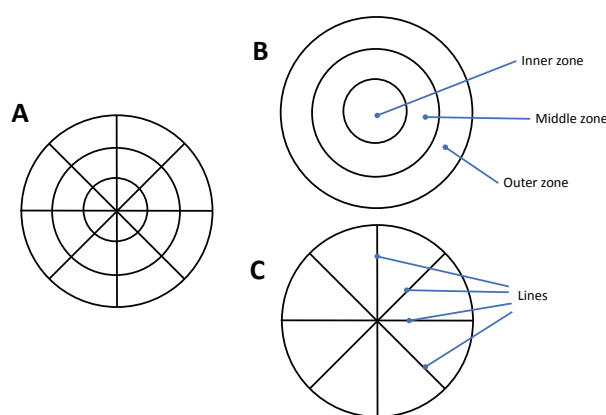


Figure 1. A diagram of markings on the experimental arena, broken down into the constituent zones (B) and lines (C). The zones were used as a determinant of thigmotactic behaviour (hypothesis 2), whereas the lines were used to determine line crosses, a measure of activity (hypothesis 3).

Table 1. Ethogram showing recorded behaviours and how they were defined by the researchers.

Behaviour category	Behaviour	Definition
Aggression	Bump	Collision between the two gammarids
	Approach	Approach of gammarid out of possession towards bloodworm held by other gammarid
	Wrestle	Both gammarids holding same bloodworm
Boldness	Outer zone	Gammarid in outer ring (Fig. 1)
	Middle zone	Gammarid in middle ring (Fig. 1)
	Inner zone	Gammarid in central ring, also initial location of bloodworm (Fig. 1)
Activity	Line cross	When gammarid crosses from one “segment” into another (Fig. 1)
Possession	In possession	Gammarid holding bloodworm
	Not in possession	Gammarid not holding bloodworm

successful at outcompeting native species for preferred habitat (Kotta et al. 2011, Reialu et al. 2016), while exhibiting high feeding rates (Dick 1996, Dickey et al. 2021) and demonstrating aggression towards native gammarids (Orav-Kotta et al. 2009, Kotta et al. 2010). As a result, we hypothesise that: 4) the trial type (i.e. species match-up) and size disparity will have significant effects on the number of aggressive interactions (i.e. bumps, approaches: see Table 1); 5) contests involving the “aggressive” *G. tigrinus* will be more likely to escalate into “wrestles” over the food resource 6) the number and duration of wrestling bouts will be significantly affected by trial type and contestant size disparity; 7) for the gammarid added first, larger *G. tigrinus* will spend greater proportions of time in possession; and 8) *Gammarus tigrinus* will be more likely to dispossess *G. duebeni*, and larger individuals will be more likely to dispossess smaller individuals.

Methods

Gammarid collection and maintenance

Both *G. tigrinus* and *G. duebeni* were collected from Travemünde, Germany (53°83'N, 10°64'E) in August 2017 and kept in laboratory conditions (temperature: 16 °C, light regime: 12 h:12 h). While the prevalence of these species from this locality were approximately 50:50 in 2017, the NNS has since become dominant and the native extirpated (Briski, pers. obs.). Stocks of both study species were held in constantly aerated 56 L glass aquaria, filled with 10 ppt, 5-µm filtered Kiel Fjord water. Sand and artificial structures in the form of ceramic tubes were added to the tanks to simulate natural habitats. The animals were fed *ad libitum* with a mixture of commercial crustacean food (Tetra Mix, Tetra Crusta, and Dr. Shrimp Healthy).

Contest trials

48 hours before trials began, study gammarids were taken from holding tanks and held individually in 100 ml of 10ppt water within plastic dishes (8.4 cm diameter, 4.2 cm height) containing two ceramic tubes for shelter. Study individuals of length 5.9 mm and upwards from both species were chosen, and randomly assigned to dyads for contests. To standardise hunger levels, and in turn motivation, gammarids were given 3 defrosted frozen *Daphnia* sp. (Vivantis Aqua, Germany) for one hour, after which any remaining *Daphnia* were removed by pipette. They were then starved for the following 47 hours. After the removal of food, all individuals were measured

and weighed within 30 minutes, so as to give ample recovery time prior to trials. For this, individuals were blotted dry and weighed, and photographed next to a ruler for scale. Using ImageJ software (Schneider et al. 2012), the head-to-telson length of each individual was measured (see Table 2 for means and standard errors, SE, and Suppl. material 1 for the size and mass measurements for each study individual).

Table 2. Mean head-to-telson lengths and masses for contestants of both species over the four experimental trial types.

Matchup	Species	Mean length (mm)	Length SE	Mean mass (g)	Mass SE
1	1. <i>G. duebeni</i>	8.356	0.600	0.051	0.006
	2. <i>G. duebeni</i>	7.912	0.399	0.043	0.006
2	1. <i>G. duebeni</i>	9.097	0.514	0.066	0.010
	2. <i>G. tigrinus</i>	8.519	0.522	0.048	0.006
3	1. <i>G. tigrinus</i>	8.958	0.339	0.061	0.006
	2. <i>G. duebeni</i>	8.189	0.478	0.048	0.005
4	1. <i>G. tigrinus</i>	8.630	0.263	0.057	0.006
	2. <i>G. tigrinus</i>	8.489	0.330	0.051	0.006

Trials were run in September 2022. Prior to trials commencing, a single defrosted frozen chironomid larva (i.e., bloodworm; Vivantis Aqua, Germany) was added to the experimental arena (same dimensions as plastic dishes mentioned above; design shown in Fig. 1). Each trial had a “first” and “second” gammarid, referring to the order in which they were added. Video recording commenced (CX Action Camera, ACTIVEON Inc., U.S.A.) and the first gammarid was added to the arena five minutes after the addition of the bloodworm. The second gammarid was added as soon as the first gammarid took hold of the bloodworm or, in the case where the first gammarid did not take hold of the bloodworm, after it had been in the arena for 20 minutes (mean \pm SE time until addition of second gammarid: 14.675 \pm 1.692 seconds). Trials ended twenty minutes after the addition of the second gammarid. There were four trial types ($n = 9$ contests per trial type, mean and SE lengths and masses of contestants outlined as per Table 2) designed to assess both intraspecific (essentially control trials) and interspecific competition as follows: 1) *G. duebeni* (first gammarid) v *G. duebeni* (second gammarid); 2) *G. tigrinus* (first) v *G. tigrinus* (second); 3) *G. duebeni* (first) v *G. tigrinus* (second); and 4) *G. tigrinus* (first) v *G. duebeni* (second).

Video analysis was conducted using BORIS v7.4.14 (Friard and Gamba 2016), with the coded behaviours falling under the categories of “aggression”, “activity”, “boldness”, and “possession” (definitions outlined as per Table 1).

Hypotheses and statistical analyses

We tested eight hypotheses, using the following statistical analyses:

1. To determine if, of the individuals that took possession, there was a difference between the two species in latency to take possession of the resource, the effect of species on latency to approach was assessed by Wilcoxon rank sum test.
2. To assess whether, of the individuals added first to the arena, there was a difference between the species in terms of thigmotactic behaviour for the period of time that they were the sole individual in the arena, we used beta regression.

The proportion of time in the outer zone (Fig. 1) was used as the dependent variable, with species and 1) head-to-telson length, or 2) mass, used as the independent variables. Dispersion was allowed to depend on the effect of species (see Cribari-Neto and Zeileis 2010).

3. To determine if there was a difference in activity between the species, we used generalised linear models (GLMs) assuming Quasipoisson error structures to account for over-dispersed data, with the number of line crosses per time spent in the arena used as the dependent variable, and focal species, opponent species and either 1) head-to-telson length, or 2) mass, used as the independent variables. Individuals added first and individuals added second were pooled for this analysis.
4. To assess the role of trial type (i.e. species match-up) and size disparity - using 1) head-to-telson length disparity or 2) mass disparity - on the number of aggressive interactions (i.e. bumps, approaches: see Table 1), we used a Quasipoisson GLM, again due to over-dispersed count data.
5. To determine if there was a significant effect of match-up on the likelihood of wrestling occurring during a contest, a 4-sample test for equality of proportions without continuity correction was used.
6. To determine if the duration of wrestling bouts was significantly affected by trial type and contestant size disparity, using either 1) head-to-telson length disparity or 2) mass disparity, on the time spent wrestling, a linear model was used. To determine the effect of trial type and size disparity on the number of wrestling bouts, we used a Quasipoisson GLM due to over-dispersed count data.
7. To test if larger *G. tigrinus*, of the gammarids added first, spent greater proportions of time in possession, we used a Quasibinomial family GLM with time in possession of the gammarid added first as the dependent variable and the trial type and 1) head-to-telson length disparity or 2) mass disparity used as the independent variables.
8. To determine if *G. tigrinus* were more likely to dispossess *G. duebeni*, and if larger individuals were more likely to dispossess smaller individuals, we used binomial GLMs with logit link functions with takeover success when *G. tigrinus* or *G. duebeni* were in possession as the dependent variable, and the species out of possession, and the 1) head-to-telson length disparity or 2) mass disparity, as the independent variables.

Statistical analyses were conducted using R v4.0.3 (R Development Core Team), with graphs created using the 'ggplot2' (Wickham 2016), 'ggpubr' (Kassambara 2023) and 'interactions' (Long 2024) packages.

Results

Gammarus tigrinus were more likely to take hold of the bloodworm in the opening 20 minutes, and those that did, did so more quickly than *G. duebeni* (hypothesis 1). Twenty-one of the individuals added first took hold of the bloodworm in the opening 20 minutes, the majority of which were *G. tigrinus* (*G. tigrinus*: $n = 15$, 71.4%; *G. duebeni*: $n = 6$, 28.6%). Of the individuals that took the bloodworm in the opening 20 minutes, *G. tigrinus* had a significantly lower latency (mean \pm SE: *G. tigrinus* 270.00 \pm 55.00 seconds; *G. duebeni* 343.23 \pm 77.36 seconds; Wilcoxon rank sum exact test: $W = 74$, $p = 0.023$).

There was a significant effect of species on the proportion of time spent in the outer zone (hypothesis 2), with *G. tigrinus* spending more time in the middle and inner zones than the native *G. duebeni* (Beta regression: $z = 5.858$, $p < 0.001$; Fig. 2; Suppl. material 1: table S1). There was also a significant two-way interaction of species and length on activity ($F_{1,67} = 5.914$, $p = 0.019$; Suppl. material 1: table S2) as measured by the number of line crosses per time spent in the arena (hypothesis 3), with *G. tigrinus* more active with increasing length, but *G. duebeni* less active with increasing length (Fig. 3A). A similar pattern was shown when mass was accounted for, with a significant two-way species and mass interaction on activity ($F_{1,67} = 6.646$, $p = 0.016$; Fig. 3B; Suppl. material 1: table S3).

With regards to hypothesis 4, there was no significant effect of trial type and head-to-telson length disparity or mass disparity on the combined number of bumps and approaches between the participant gammarids (Quasipoisson GLM: trial type the sole independent variable in both minimum adequate models, $p = 0.249$). However, at an individual level, a significant two-way interaction effect between species and mass on the number of approaches and bumps (Quasipoisson GLM: $F_{1,68} = 5.202$, $p = 0.040$; Suppl. material 1: table S4), with larger *G. tigrinus* committing more bumps and approaches, but larger *G. duebeni* committing fewer (Fig. 4).

There was no significant effect of trial type on proportion of contests that led to wrestles (4-sample test for equality of proportions without continuity correction: $p = 0.517$; hypothesis 5). Similarly, there were no significant effects for trial type and head-to-telson length disparity (Gaussian GLM: length disparity the sole independent variable in minimum adequate model, $p = 0.165$) or mass disparity

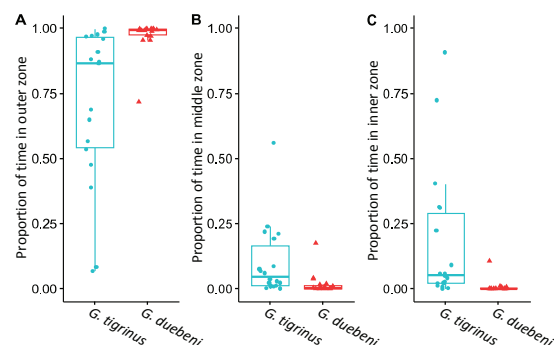


Figure 2. Boxplots outlining the relative proportional time spent in each of the three zones and how this differed between *G. tigrinus* and *G. duebeni* **A** outer zone **B** middle zone **C** inner zone.

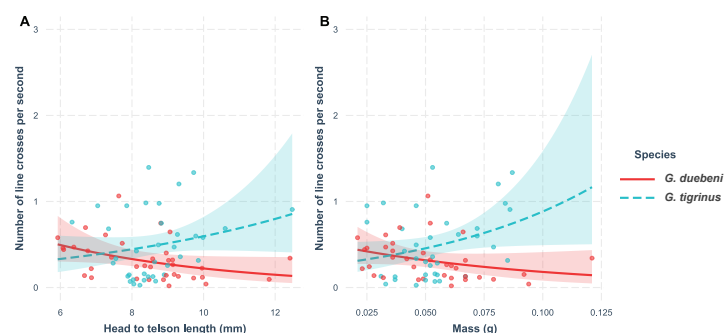


Figure 3. Scatter plots showing the effects of **A** mass and **B** head-to-telson length on the number of line crosses per second in the arena, and how this differed between *G. tigrinus* and *G. duebeni*. Lines shown with 95% confidence intervals.

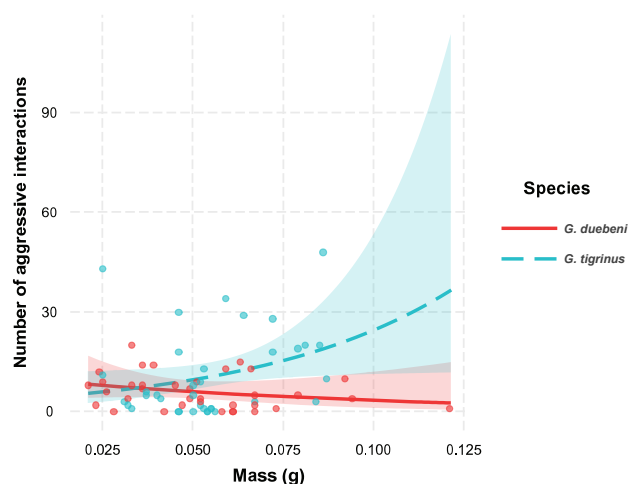


Figure 4. Scatterplot outlining the effect of mass on the number of aggressive interactions, and how this differed between *G. tigrinus* and *G. duebeni*. Lines shown with 95% confidence intervals.

(Gaussian GLM: trial type the sole independent variable in minimum adequate model, $p = 0.153$) on the log-transformed time spent wrestling or the number of wrestling bouts (Quasipoisson GLM: trial type the sole independent variable in both minimum adequate models, $p = 0.115$; hypothesis 6).

There were no significant effects of trial type and size disparity or mass disparity on the proportion of time in possession found for the gammarid added first (Quasibinomial GLM: trial type the sole independent variable in both minimum adequate models, $p = 0.230$; hypothesis 7). However, takeover success when *G. tigrinus* was in possession was significantly affected by the species out of possession (Binomial GLM: $z = 2.249$, $p = 0.025$; Suppl. material 1: table S5), with *G. tigrinus* taking possession significantly more than *G. duebeni* (Table 3; hypothesis 8). Takeover success when *G. duebeni* was in possession was not significantly affected by the species out of possession (Binomial GLM: the species previously not in possession was the sole independent variable in minimum adequate model, $p = 0.205$).

Table 3. Takeover attempt success based on species attempting takeover and species in possession at the time.

Species in possession	Opponent attempting takeover			
	<i>G. tigrinus</i>		<i>G. duebeni</i>	
	Successful	Unsuccessful	Successful	Unsuccessful
<i>G. tigrinus</i>	4	1	1	8
<i>G. duebeni</i>	2	2	2	5

Discussion

The introductions of NNS with overlapping ecological niches and functional similarity can lead to displacement of native species, something that has repeatedly been documented for those faced with the arrival of *G. tigrinus* (Pinkster et al. 1992, Jänes et al. 2015, Reialu et al. 2016). In this study, we staged dyadic contests, using four combinations featuring the NNS and the native *G. duebeni*, over a single food resource, whereby one contestant was allowed up to twenty minutes to take possession of the resource undisturbed, with a second contestant added once the first contestant took possession, or after twenty minutes had elapsed. We found *G. tigrinus* to exhibit

behaviours deemed to be beneficial across multiple stages of the invasion process and to exert a competitive advantage over the native gammarid which, through limiting access to resources, could affect population growth and survival.

During the initial period, we found *G. tigrinus* to be more likely to take possession of the resource, and of the individuals from both species that took possession, *G. tigrinus* exhibited a lower latency to do so than *G. duebeni*. The ability to efficiently identify, locate, take and retain possession of food resources is a valuable way of overcoming competitors in a novel ecosystem. Lower latencies to commence foraging have also been linked to greater boldness (Short and Petren 2008), and indeed, during this same period (i.e. before the addition of the second individual), *G. tigrinus* spent a lower proportion of time in the outer zone, indicating less centrophobic or thigmotactic behaviour than the native *G. duebeni*. Thigmotactic behaviour is a common indicator of where an individual falls along a boldness-shyness axis (dos Santos et al. 2023, Augustyniak et al. 2024), and boldness is a trait offering benefits at different stages of the introduction process. While some behaviours can have mixed effects depending on the stage (e.g. exploratory behaviour might enhance the likelihood of uptake into transport vectors but increase the likelihood of detection by biosecurity checks during transit: Chapple et al. 2011), boldness is thought to provide benefits during uptake, introduction, establishment and spread (Chapple et al. 2012, Gruber et al. 2018). Boldness is also often correlated with other “dispersal-enhancing traits” (Gruber et al. 2018, McGlade et al. 2022) such as activity and aggression within a behavioural syndrome (Sih et al. 2004), and the combination of high boldness and activity levels has been shown to enhance feeding opportunities (Brownscombe and Fox 2013) and survival in the presence of predators (Blake et al. 2018). We also discovered size-dependent activity and aggression differences between the species, with larger *G. tigrinus* found to be more active and aggressive, but larger *G. duebeni* found to be less so. While this aggression did not equate to increased success of dispossessing the native *G. duebeni*, which were equally likely to concede possession to conspecifics as they were to *G. tigrinus*, it may have offered some sort of deterrent, with *G. tigrinus* being better at resisting takeover attempts from *G. duebeni* than from other *G. tigrinus* individuals.

The inability of *G. duebeni* to dispossess *G. tigrinus* when in possession could be explained by competitive naiveté, something that remains relatively unstudied (Heavener et al. 2014). It may be that *G. duebeni* adapt to *G. tigrinus* as a competitor over time, but this is hard to predict, with some behavioural changes instant, some occurring over an individual’s lifetime, and some over multiple generations (Ruland and Jeschke 2020). Furthermore, considering the rates at which native gammarids have been replaced by *G. tigrinus* (e.g. Kazanavičiūtė et al. 2024), such adaptive processes may be too lengthy. For example, Heavener et al. (2014) showed that despite a close taxonomic relationship, native bush rats (*Rattus fuscipes*) failed to recognise the chemical cues of non-native black rats (*Rattus rattus*) in Australia, despite the two species interacting competitively for over 200 years. However, adaptation periods can be much shorter. The NNS American mink (*Neovison vison*) established in the UK at a time that otters (*Lutra lutra*) and polecats (*Mustela putorius*), both native competitors, were largely absent. Since then, populations of the native species have recovered and expanded, and mink have changed from being nocturnal to diurnal over the course of a decade, which is theorised as an adaptation to the rebounding natives (Harrington et al. 2009). It remains to be

seen if *G. duebeni* can adapt competitively and quickly enough, or whether they can expand the niche differentiation with *G. tigrinus* through resource partitioning or avoidance, either in space or time.

In the present study, we did not see any significant effect of body mass or length on contest outcome or aggressive behaviour between *G. tigrinus* and *G. duebeni*. Those size measures are commonly used determinants of competitive ability or “resource holding potential” (Arnott and Elwood 2009, Zeng et al. 2019), and would be expected to influence contest dynamics. It could be that the size disparities and sample sizes between our competing species might have been too small to find such an effect, and it is an area that warrants further investigation in future studies. During contests, individuals are thought to assess their own and their opponent’s resource holding potential as well as the value of the contested resource, and this can dictate whether individuals risk injurious and potentially deadly fights (Arnott and Elwood 2008). In addition to morphological adaptations, which we propose are less relevant for gammarids, it may be that some species attach a naturally higher value to a contested resource than others, potentially driven by metabolism, or having more specialist demands in terms of diet or habitat. Other studies have also found species to be the most important predictor, rather than size. For example, this was shown for sympatric salamanders (Anthony et al. , 1997) and rockpool fishes (Paijmans and Wong 2017).

While the purpose of this study was to assess species-level differences, the methods employed could also be used to compare the competitive abilities of age-groups, sexes or populations. Indeed, there have been a number of calls to assess population-level differences within invasion ecology of late (Haubrock et al. 2024) and such differences have been noted in our study species. For example, individuals from the Dutch, Lough Neagh-originating *G. tigrinus* population are deemed less adapted to freshwater conditions than those from the German, England-originating population which subsequently invaded the eastern areas of the Netherlands (Pinkster et al. 1992). Therefore, applying dyadic contests across a salinity spectrum could reveal the environmental tipping point where one population is at a competitive advantage. Another avenue for study could be to look at the North American native range of *G. tigrinus*, where it is represented by six genetically distinct lineages grouped in two main clades (the “northern species” and “southern species”: Kelly et al. 2006). With the European populations descended from the “northern species” (Rewicz et al. 2019), it would be of interest to see how these clades compare, and whether differences in behaviour make one clade more prior-adapted to exert potential impacts. More generally, such a population focus can allow our method to help test invasion ecology hypotheses like the invasion front hypothesis (Lopez et al. 2012, Iacarella et al. 2015) or anthropogenically induced adaptation to invade (Hufbauer et al. 2012, Briski et al. 2018).

An important next step is to ground-truth the methods applied in this study and to use more real-world examples. While size seemed to play a minor role in our study, we note that adult *G. duebeni* can reach almost twice the size of adult *G. tigrinus* (Kolding 1981, Ward 1985, Grabowski et al. 2007), and we recommend that future studies account for larger individuals to determine their competitive performance and if the trend of decreasing activity and aggression holds. While size disparity can lead to different habitat utilisation and in turn segregation and coexistence (e.g. Platvoet et al. 2009), there are many case studies where much larger gammarid NNS have displaced smaller natives (however, conversely, native

G. lacustris has been displaced by the physically smaller *G. fasciatus* in Lake Peipsi in Estonia and Russia: Panov et al. 2000, Panov and Berezina 2002). One such example is the larger Ponto-Caspian NNS *D. villosus* (adult males can reach 30 mm body length: Neumann et al. 1995), which has had negative impacts on *G. tigrinus* and *G. duebeni* populations in the Netherlands (Dick and Platvoet 2000). Size disparity may also heighten the risk of contests descending into intraguild predation (Polis et al. 1989), and asymmetrical mutual predation has been shown to be a key driver of NNS replacing natives (Dick and Platvoet 1996, Nakata and Goshima 2006). Staging dyadic contests with large interspecific size disparities could help reveal the prevalence of this phenomenon.

Going forward, we propose that the findings of this study, and the methods implemented to derive them, offer applied potential in the form of NNS impact assessment. Indeed, recent impact assessment measures comparing resource consumption rates between NNS and trophically analogous natives have become incredibly popular within invasion ecology (Dick et al. 2014, Faria et al. 2023, 2025). Nevertheless, the *per capita* nature of the method, with study individuals left to feed in the absence of conspecific or interspecific competitors (Dickey et al. 2022), is an obvious shortcoming that could be addressed using the method at hand. Indeed, while the functional response-derived Relative Impact Potential metric (Dick et al. 2017, Dickey et al. 2020) has incorporated proxies for the numerical response to compare the population impacts of NNS relative to native species, it could be further developed to include a measure of competitive ability (or conversely, the degree of biotic resistance posed by native species within an ecosystem: Twardochleb et al. 2012, MacNeil et al. 2013), leading to a measure of potential impact across three axes. This would determine: 1) the “undisturbed” maximum feeding rate relative to a native, 2) the potential population size or reproductive rate of a NNS relative to a native, and 3) a measure of interspecific competitive strength to determine the probability of the two prior axes being realised. All three measures will likely change depending on abiotic conditions (e.g. salinity, temperature, noise pollution), and these can in turn be accounted for, allowing, for example, the prediction of future impacts with climate change. These three axes would account for many of the myriad mechanisms and traits that have been suggested as explaining the invasion success and impacts of *G. tigrinus* to date. For example, the NNS exhibits a broad reproduction period, known to be reproductively active all year round in the Gulf of Riga (Kotta et al. 2010), as well as a high reproduction rate, a short development time, high feeding rates with a propensity for “surplus killing” of prey (Dickey et al. 2021), and broad ecological tolerances in terms of salinity, temperature, and pollution (Grabowski et al. 2007, Reialu et al. 2016).

In summary, we propose that this method, used here to demonstrate the competitive ability of a widespread NNS that has led to population declines of native gammarids across Europe and is expanding its range further (Rewicz et al. 2019), can offer a standardised, effective means of assessing inter- and intraspecific contests over limited resources. Bridging the gap between animal behaviour studies and applied NNS impact assessment, we propose it can be easily tailored depending on the hypotheses and study systems in question. While more ground-truthing is required, we believe it has the potential to become a useful component of horizon scans, and thus facilitate the impact assessment, prediction, and prioritisation of NNS as required by EU legislation and global biodiversity targets.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.


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Author contributions


JWED and GA conceived the study. JWED conducted the experiments. JWED and JWZ performed the video analyses and conducted statistical analyses. EB and JMJ provided resources. JWED led the writing of the manuscript, with all authors contributing to its development.

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Data availability

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

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Supplementary material 1

Supplementary information

Authors: James W. E. Dickey, Julian W. Zeller, Elizabeta Briski, Jonathan M. Jeschke, Gareth Arnott

Data type: docx

Explanation note: **table S1.** Estimated regression parameters, standard errors, z values and p -values for the Beta regression. The proportion of time spent in the outer zone was used as the dependent variable, with species and 1) head-to-telson length, or 2) mass, initially used as the independent variables, however the minimum adequate model was left with only species remaining following stepwise deletion. Dispersion was allowed to depend on the effect of species. **table S2.** Estimated regression parameters, standard errors, t values and p -values for quasipoisson generalized linear model. The number of line crosses per time in arena was used as the dependent variable and species of the focal individual, species of its competitor, and head-to-telson length were used as the independent variables. **table S3.** Estimated regression parameters, standard errors, t values and p -values for quasipoisson generalized linear model. The number of line crosses per time in arena was used as the dependent variable, and species of the focal individual, species of its competitor and mass were used as the independent variables. **table S4.** Estimated regression parameters, standard errors, t values and p -values for quasipoisson generalized linear model. The number of aggressive interactions (i.e. bumps, approaches: see Table 1) was used as the dependent variable, with species and mass used as independent variables. **table S5.** Estimated regression parameters, standard errors, z values and p -values for binomial generalized linear model with logit link. Take-over success when *G. tigrinus* were in possession was used as the dependent variable, with the species out of possession, and the 1) head-to-telson length disparity or 2) mass disparity, used as the independent variables. After stepwise removal of terms, the species out of possession was left as the sole independent variable in the minimum adequate mode.

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Link: <https://doi.org/10.3897/neobiota.97.141901.suppl1>

Supplementary material 2

Gammarid contest data

Authors: James W. E. Dickey, Julian W. Zeller, Elizabeta Briski, Jonathan M. Jeschke, Gareth Arnott

Data type: xlsx




Explanation note: Behaviour data, coded using BORIS.

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Short Communication

Advancing the Risk Analysis for Alien Taxa (RAAT) framework

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Abstract

The Risk Analysis for Alien Taxa (RAAT) framework was developed to collate scientific information and interpret it in the context of South African regulations on biological invasions. Feedback from the past four years has shown RAAT to be a valuable tool for making evidence-based, transparent recommendations, but one that can be improved. We describe the changes made in creating RAAT v2.0. We note several issues that we suspect will apply to many similar processes, namely the need to i) clarify descriptions; ii) remove superfluous questions; and iii) add questions to fully justify recommendations. We also stress what RAAT v2.0 does not do—the framework does not summarise management best practices nor does it, or South Africa's regulatory lists, provide sufficient information to prioritise management resources. We strongly recommend that explicit feedback mechanisms are set up to ensure that similar such frameworks can be improved over time.

Key words: Biological invasions, decision making, regulations, risk assessment

Introduction

Risk analyses for alien taxa cover many aspects of biological invasions, such as the likelihood of introduction, establishment, spread, and impacts, management options as well as risk communication. Risk analysis frameworks are essential for the development of evidence informed regulation of alien species, with many new frameworks published in recent years (Sankaran et al. 2023). While some general standards for risk analyses have been identified (Roy et al. 2018), different regulatory frameworks often have slightly different requirements, which can explain the large variety of frameworks available (e.g., Verbrugge et al. 2010; Kumschick and Richardson 2013). For example, the European Union (EU) Regulation on Invasive Alien Species considers a selection of invasive species, all of which must be controlled in all member states (European Union 2014). In South Africa, however, the general approach is to list all invasive species that pose a risk of causing harm, with specific details of how to control taxa outlined in national management plans (Department of Environmental Affairs 2020), i.e., taxa are listed regardless of whether control programmes are likely to be feasible or effective. Regardless of the exact purpose of a risk analysis framework, it is important that the framework is evaluated, tested, and amended based on lessons learnt during its application. In particular, users can uncover inconsistencies, or highlight aspects that might be



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ambiguously worded; the science underpinning the frameworks advances; and the regulatory requirements might change (e.g., Weed Risk Assessment table in Kumschick and Richardson 2013; Kumschick et al. 2024).

South African regulation of alien species

In South Africa, the National Environmental Management: Biodiversity Act (NEMBA, Act 10 of 2004) Alien and Invasive Species Regulations (hereafter called the NEMBA A&IS Regulations; Department of Environmental Affairs 2020), list alien taxa that need to be managed. It separates taxa into different categories: Category 1a regulated taxa are considered nation-wide eradication targets; category 1b listed taxa require control; category 2 taxa can be used with permits under certain conditions and are 1b outside of permitted uses; category 3 taxa are exempt for certain uses but otherwise need control (i.e., somewhat similar to category 1b and 2, but no need for permits to keep existing individuals). There is also provision for a list of prohibited taxa, i.e., taxa which are not yet present and may not be imported into the country, although such a list was not included in the 2020 version of the regulations. For a detailed discussion of the NEMBA A&IS Regulations and the listing categories see Wilson and Kumschick (2024).

Development of the RAAT framework and application

The Risk Analysis for Alien Taxa (RAAT) framework was developed to provide robust, scientifically based evidence to support the listing of alien taxa under the NEMBA A&IS Regulations. The framework provides a structured, transparent approach for the evaluation of the risks a taxon poses. The first version of RAAT (v1.0) was developed in 2017 in response to the need from government for a transparent framework to underpin the regulation of alien species. The framework underwent minor updates based on the initial experiences and was published as a preprint in 2018 (v1.1). In 2020, a trialled, tested, and revised version of RAAT was peer-reviewed and published in *NeoBiota* (Kumschick et al. 2020; see Suppl. material 1: table S2 for discussion on all the versions of RAAT to date). The framework has since been applied to various taxa as published in the scientific literature (Keet et al. 2020; Canavan et al. 2021; Matthys et al. 2022; Mbobo et al. 2022), and, as of 30 April 2024, has been applied to 123 of the 560 taxa regulated in South Africa and 17 taxa that are not currently regulated (Wilson and Kumschick 2024).

In many cases, assessors, reviewers, and the Alien Species Risk Analysis Review Panel [ASRARP, an independent scientific body which oversees the review process, see Wilson and Kumschick (2024) for details], broadly agreed both with the scoring of particular sections and with the final recommendations. Changes suggested during review were almost always agreed upon and the additional information provided a more solid basis on which recommendations were built. The main exception was that conflict taxa (i.e., taxa which cause negative impacts through invasions but have benefits to some sectors of society), could often not be confidently assigned to a regulatory category. Conflict taxa in South Africa include invasive freshwater fishes used for recreational angling that have caused the extirpation of native fish populations through predation, animals used as pets that can escape captivity and pose a threat to native species, and various ungulates introduced to

game farms that can pose a threat to the genetic integrity of native taxa (see also Zengeya et al. 2017). Additional information was also deemed necessary to justify why certain exemptions were specified in the regulations or to justify conditions under which permits could or could not be issued. Furthermore, some information requested by the RAAT framework (e.g., on management best practice) was found not to affect the decision of whether and how to list a taxon. Certain sections were also not easily applied across taxonomic groups [e.g., propagule persistence can be critical to determine the feasibility of eradicating an invasive plant population (Panetta and Timmins 2004), but is less meaningful for many animals]. Therefore, after more than two years of applying and testing the framework, we re-evaluated the RAAT. This re-evaluation was based on the experience conducting assessments, reviewing assessments as part of the ASRARP, and teaching courses on how to apply RAAT (the later by S Kumschick).

Here we provide an overview of the main issues and gaps encountered with v1.2 of the RAAT, how they are addressed in v2.0, and flag general issues that might be useful for those who are revising similar such risk analysis frameworks.

Changes made between RAAT v1.2 and v2.0

We identified three broad issues: i) the need to clarify some descriptions in the guidelines; ii) some information requested was superfluous to classifying taxa or developing recommendations; and iii) additional information was needed to justify the recommendations. The following paragraphs present an overview of these issues, with details of the changes made between RAAT v1.2 and v2.0 (for full details see Suppl. material 1).

i) Clarifications of descriptions

The guidelines for RAAT v1.2 were published as an appendix to a scientific paper which contained details required for the assessments (Kumschick et al. 2020), however we found that many of the issues discussed in detail in the scientific paper (but not in the guidelines) were not routinely addressed by those conducting risk analyses. To ensure the documentation was consolidated and more readily accessible, all the relevant information was added to the guidelines document (see Suppl. material 1: table S2 for links to documents on Zenodo). Furthermore, some questions and response options were frequently misinterpreted and needed to be rephrased to reflect their true intention. For example, the term “propagules” is mainly used for plants, but given the cross-taxon nature of RAAT it needed to be clarified for other taxa (e.g., life cycle stages). Also, it was clarified that information on impacts of congeners should only be included if justified by their similarities to the taxon.

ii) Information removed

RAAT v1.2 included a section with four questions on life history traits and other factors which could determine how easy the management of the taxon would be. While these questions are relevant for developing management plans and identifying eradication targets (Wilson et al. 2017), they are less relevant for deciding the appropriate listing category under South African regulations, and thus they were removed. Often only through a detailed evaluation of management feasibility (that

will require some trial management) can a robust decision be made on the most appropriate management goal (in particular whether eradication is feasible). We strongly support calls for adaptive management (Zengeya and Wilson 2023)—a lack of information should not be an excuse for inaction and decisions should also be updated as new information is collected as part of control operations. The aim of RAAT is to support listing decisions, and so the information presented is a very small and select part of that needed to prioritise management resources. It is important to stress that the RAAT should not be used as a primary tool for management prioritisation. The RAAT is a tool to collate information needed to decide between broad management goals (i.e., Is regulation needed? Has an analysis explored the feasibility and desirability of attempting eradication? And are there any contexts where exemptions or permitting might be appropriate?).

iii) Information added

ASRARP, in their role of reviewing risk analyses before they are submitted to the department responsible for the NEMBA A&IS Regulations (see also Wilson and Kumschick 2024), often requested that further information be added in cases where exemptions were recommended, or where benefits were identified and permits allowed. In RAAT v1.2 a section was included where assessors could provide further information necessary to make decisions. As it was not clear what was needed, this section was often left blank. A new structured section on recommendations has been added to RAAT v2.0 to ensure the justification behind specific recommendations is clear. The information requested on the benefits of a taxon is also more explicit, asking for, amongst other things, which stakeholders are benefitting. Finally, a section was added to the guidelines on general formatting and rules to improve consistency between assessments.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.


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Author contributions

All authors contributed to the conceptualisation of the manuscript. SK led the development of the framework and writing, with inputs from JR UW and LCF.

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Data availability

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

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Supplementary material 1

Supplementary information

Authors: Sabrina Kumschick, Llewellyn C. Foxcroft, John R. U. Wilson

Data type: docx



Explanation note: **figure S1.** An overview of the Risk Analysis for Alien Taxa (RAAT) framework v2.0. **figure S2.** Decision tree for making recommendations for listing categories (1a, 1b, 2) of alien taxa as per South Africa's NEMBA A&IS Regulations. **table S1.** Changes made between v1.2 and v2.0 of the Risk Analysis for Alien Taxa (RAAT) framework. **table S2.** Versions of the Risk Analysis for Alien Taxa (RAAT) framework available on Zenodo.

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Research Article

Intrapopulation differences in biological traits and impacts in a highly invasive freshwater species

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Abstract

Individual variation assessments are essential to better understand population and community dynamics, as well ecosystem functioning. Although researchers have long recognized this aspect, only recently has evidence accumulated about the ecological importance of variation within species. The incorporation of individual variation provides an even more complete description of the effects a species may have on ecosystems and this detailed ecological knowledge can be especially important in the context of biological invasions. In this study, we used an invasion gradient of the signal crayfish *Pacifastacus leniusculus* in the Rabaçal River (NE Portugal) to assess possible intrapopulation differences in key biological traits and evaluate possible changes in ecological impacts. For this, we collected individuals from the core and front of an invasion gradient to characterize several traits such as abundance, size, sex-ratio, body condition, behaviour (i.e. boldness), and trophic niche. In addition, we performed two laboratory experiments to assess possible differences regarding the consumption of prey (gastropods), leaf mass loss, and nutrients release. Signal crayfish from the front of the invasion gradient have lower abundance, are larger, predominantly male, have better body condition in both sexes, exhibit increased boldness, and have higher $\delta^{15}\text{N}$ and lower $\delta^{13}\text{C}$ values. In addition, in experimental conditions, signal crayfish from the front of the invasion gradient consumed more gastropods and leaves and increased the concentration of nitrates and phosphates in the water. Overall, the signal crayfish has different biological traits and distinct ecological impacts along the invasion gradient in the studied river. Our study demonstrates the relevance of assessing biological traits and impacts of invasive species at the intrapopulation level.



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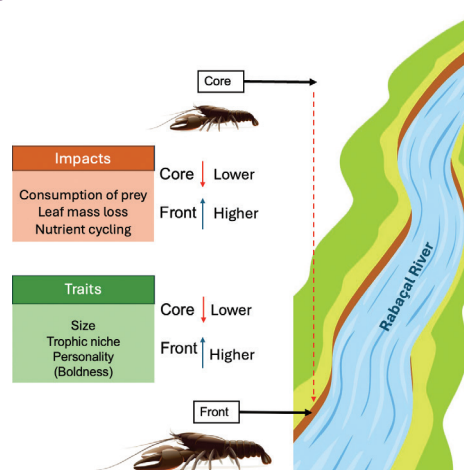
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Graphical abstract



* Both authors contributed equally as the first author.

Key words: Biological traits, ecological impacts, invasive species, *Pacifastacus leniusculus*, signal crayfish

Introduction

Humans are key drivers of global environmental change (Dirzo et al. 2014) and anthropogenic activities have redistributed the world's biota and mediated species colonization of regions beyond their native range (Seebens et al. 2017; Pyšek et al. 2020). The consequences of these biological introductions are severe. Non-native species can disturb ecological communities and alter ecosystem functions, drive population declines and species extinctions, and continue to cost the global economy millions of euros every year given their detrimental negative impacts on several key ecosystem services (Ehrenfeld 2010; Strayer 2012; Simberloff et al. 2013; Diagne et al. 2021; Gallardo et al. 2024). Given the myriads of detrimental ecological, economic and social impacts attributed to non-native species and limited possibilities for total eradication, it is not surprising that their study is a prominent area of research (Pyšek et al. 2020). Although all ecosystems are susceptible to species introductions, freshwater ecosystems are especially vulnerable (Strayer 2010). This situation presents a challenge for the management and conservation of freshwater biodiversity because accelerated introduction rates may have detrimental consequences, including the erosion of biodiversity and as such, the disruption of key ecological processes and functions (Strayer 2010; Gallardo et al. 2016; Dudgeon 2019).

Understanding the species-level traits associated with invasiveness (defined in relation to arbitrary chosen quantitative metrics, which may encompass population abundance, geographical spread and ecological or socioeconomic impacts; Oficialdegui et al. 2024) has been a primary focus of biological invasions studies, with attributes such as high dispersal rates, high fecundity, and broad physiological tolerance among the key predictors of success (Pyšek et al. 2020). However, this may be an oversimplification (i.e. assessing the impacts, spread, or other topics at the species level) because a species can be very invasive in one region while simultaneously being almost innocuous a few kilometers away (Sousa et al. 2024). Moreover, a growing accumulation of evidence is showing that most of these impacts happen at the population level, not only due to the environmental context or biotic resistance of the invaded ecosystem (Blackburn et al. 2011), but also because intraspecific variation is increasingly recognized as an important driver of invasion dynamics (Biro and Stamps 2008; Haubrock et al. 2024).

In fact, environmental conditions can significantly influence the dynamics of biological invasions, including the establishment and spread of non-native freshwater species. Habitat structure, resource availability, and abiotic factors such as temperature, water quality, and flow regime can create heterogeneous landscapes that affect the success and impact of non-native populations (Lopez et al. 2022; Boon et al. 2023). Understanding how these environmental variables interact with biological traits is crucial for predicting invasion dynamics and impacts. It is likely that density-dependent processes, biotic factors (e.g. predation, competition and parasitism) and seasonal variability in abiotic factors interact to influence the life history traits observed at different stages of the invasion process (Bøhn et al. 2004). Given these idiosyncrasies, individual variation in ecological assessments is essential to understanding population and community dynamics, and ecosystem functioning (Bolnick et al. 2011; Violle et al. 2012; Des Roches et al. 2018; Raffard

et al. 2019). Although researchers have long recognized this aspect (e.g. Charles Darwin and Russel Wallace (Darwin and Wallace 1858) use these intraspecific variations as the central foundation of the theory of evolution), evidence of the ecological importance of variation within species has only recently accumulated (Post et al. 2008; Des Roches et al. 2018; Raffard et al. 2019). This last aspect may be related to variation in biological traits (e.g. size, sex, boldness, sociability, activity, aggression) and associated behavioural syndromes, i.e. correlations between these traits (Chapple et al. 2012). For example, personality, defined as individual differences in behaviours that are stable over time and context (Sih et al. 2004), can affect any stage of the invasion process (Juetter et al. 2014). This is because traits that maximize the probability of being introduced outside the native range may also promote successful establishment and spread within the recipient ecosystem (Myles-Gonzalez et al. 2015; but see Chapple et al. 2012 for discussion on possible exceptions). These biological traits are also likely to influence interactions with native species and ecosystems and thus may play an important role in determining the intensity of ecological impacts (Juetter et al. 2014). Despite the theoretical relevance, the fact is that very few studies have demonstrated how variation in biological traits may affect ecological impacts of a non-native species along an invasion gradient (Cote et al. 2010). However, biological traits, such as size, sex, body condition, and behaviour may act as important drivers on the spread dynamics of non-native species (Phillips et al. 2006) and may be responsible for distinct ecological impacts (Cote et al. 2010). For example, a study by Fryxell et al. (2015) showed that effects of a non-native freshwater fish (western mosquitofish *Gambusia affinis*) on phytoplankton and zooplankton abundance, as well as productivity, were influenced by variations in sex ratio and sexual dimorphism. Finally, these biological traits and impacts may vary not only spatially but also through time and this may differentially affect native communities over the years and may even have evolutionary consequences for native biodiversity (Bøhn et al. 2004; Mathers et al. 2016; Carvalho et al. 2022; Haubrock et al. 2024).

Therefore, it seems reasonable to assume that the incorporation of individual variation provides a more real and complete description of the population, community, and ecosystem being studied. Their downplay in empirical studies is probably related to the increased complexity of the analyses and due to additional workload or costs in laboratory and/or field sampling (Toscano et al. 2016; Sousa et al. 2024). Even so, a number of studies already demonstrate the importance of these intraspecific peculiarities in biological invasions, with the cane toad *Rhinella marina* invasion in Australia as the iconic textbook example (Shine, 2010). In this study, we use the recent invasion of the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) in a Portuguese river to assess possible differences in biological traits and ecological impacts at the intrapopulation level. This species is native to North America and was first introduced in Europe, primarily in Scandinavia, in the 1960s for stocking purposes and replacement of the native crayfish species, which suffered great declines due to crayfish plague (Dunn 2012). *P. leniusculus* is characterized by high reproductive rates, fast-growing populations, wide environmental tolerance, high aggressiveness, and high dispersal abilities, making it a very successful invader in Europe (Dunn 2012). This species is also an ecosystem engineer, altering community structure, water quality, and nutrient dynamics (Carvalho et al. 2022). It is an omnivorous species and displays generalist and opportunistic feeding habits (Olsson et al. 2009). Ultimately, the signal crayfish may

play a crucial role in invaded ecosystems, holding a central position in food webs, where it functions both as predator and prey for many taxonomic groups, affecting different trophic levels and being able to have top-down and bottom-up cascading effects (Guan and Wiles 1998; Britton et al. 2017; Meira et al. 2019).

Given its high abundance and widespread distribution, the signal crayfish is responsible for several ecological and economic impacts on invaded ecosystems, but these impacts may be highly context-dependent even considering the same population. Therefore, the main aim of this study was to assess possible intrapopulation differences in key biological traits (abundance, size, sex-ratio, body condition, behaviour, and trophic niche) and ecological impacts (consumption of prey, leaf mass loss, and nutrients release) using the signal crayfish along an invasion gradient. Analogous to previous studies (e.g. Phillips et al. 2006; Brandner et al. 2013; Rebrina et al. 2015) we hypothesize that (1) there will be significant differences in abundance, size, body condition, and sex ratio between the core and the front of the invasion gradient; (2) signal crayfish individuals at the front of the invasion will exhibit bolder behaviour compared to those in the core area; and (3) these intrapopulation differences will result in varying per capita ecological impacts, with signal crayfish at the front of the invasion gradient consuming more gastropods and leaves, and contributing to higher nutrient concentrations.

Material and methods

Study area

This study was carried out in the Rabaçal River, at the Montesinho Natural Park and adjacent downstream areas (Fig. 1), located in NE Portugal. This river has a total length of 88 km, and its hydrological basin is subjected to a typically Mediterranean climate with an Atlantic influence, characterized by high seasonal variability in temperature and precipitation (Oliveira et al. 2012). This variability in precipitation (with annual rainfall ranging between 1000 and 1600 mm) is responsible for abrupt alterations in river flow, with maximum values being registered during winter/early spring and minimum values in late summer/early autumn (Sousa et al. 2018).

The low human density in the Montesinho Natural Park, as well as the land use mainly related to forest and subsistence agricultural activities (Nogueira et al. 2021a), makes this hydrological basin one of the least disturbed in Portugal, containing important habitats for several threatened freshwater species such as the pearl mussel, *Margaritifera margaritifera*, the dragonfly *Macromia spendens*, the Northern Iberian spined loach *Cobitis calderoni*, the Iberian desman *Galemys pyrenaicus*, among many others (Sousa et al. 2015, 2019, 2020). However, in the last two decades, the populations of these threatened species suffered a reduction in abundance due to the impacts of droughts, habitat loss and fragmentation, and the introduction of non-native species, such as the signal crayfish (Sousa et al. 2019, 2020; Nogueira et al. 2021b; Lopes-Lima et al. 2023). In Portugal, the signal crayfish was first detected in 1997 in the Maças River (NE of Portugal), the main tributary of the Sabor River (Bernardo et al. 2011; Anastácio et al. 2019). Over the following years, the species rapidly spread to almost the entire Sabor basin (Meira et al. 2019). In the Rabaçal River, the species was first detected in the core site (Fig. 1) in the summer of 2013 and from there spread exclusively in the downstream direction (Sousa et al. 2015; Carvalho et al. 2024). The exact date of introduction is unknown, and

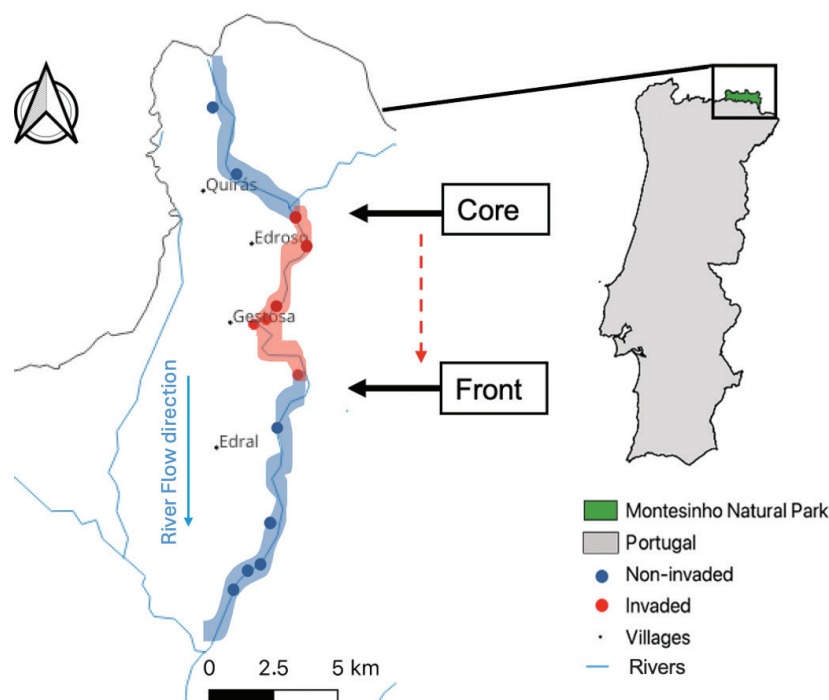


Figure 1. Map of the Rabaçal River with the location of both sites (core and front) sampled in this study. Marked dots in blue (non-invaded sites until 2023) and red (invaded sites) correspond to sampling sites monitored since 2017. The red dashed arrow indicates the spread direction of the signal crayfish.

it is believed that it was the result of one or more intentional introductions. Since 2017, this population has been monitored annually to assess their spread (Carvalho et al. 2024). This annual monitoring allows us to confidently establish the core (i.e. the original establishment site of the signal crayfish) and front (i.e. the leading edge of the signal crayfish invasion) of the invasion gradient (around 15 km distance between both sites) in the present study (Fig. 1). It should be noted, that besides the recent introduction of the signal crayfish, there are no records of any native or non-native crayfish species in the studied area (Sousa et al. 2019).

Environmental characterization

In both core and front sites (Fig. 1), in August 2023, we selected a river stretch of 100 m that comprised habitats including pools, runs, and riffles. In each site, temperature, conductivity, dissolved oxygen, and pH were measured using a YSI EXO 2 multi-parameter probe. Water samples were also collected to determine total suspended solids (TSS) as well as total organic suspended solids (OSS). For this, water volumes of 1 L were filtered using GFC filters, which were dried at 60 °C for 48 h and then heated in a muffle at 550 °C for 8 h. TSS and OSS were determined by weight difference following Zieritz et al. (2018). These measurements and water samples collections were made in the middle of the river near to the bottom at the end of the morning.

One sediment sample was also collected in each site, approximately 2 m from the left bank, using a cylinder with 10 cm of diameter. Granulometry and organic matter in the sediment of each site were determined following Sousa et al. (2007).

Both sites were also subjected to an in situ River Habitat Survey (RHS) to collect information related to the physical structure, including the complexity of the structure of the riparian vegetation, the type of aquatic vegetation, the characteristics of

the habitat and the type of artificial modification found in the channel and on the banks. For this, the standard length of 500 m of the river channel was used for data collection following Raven et al. (1998). All this collected information was used to obtain the Habitat Modification Score (HMS) and Habitat Quality Assessment (HQA) indices, which were important for ascertaining the degree of disturbance in both sampling sites (Raven et al. 1998).

Assessment of biological traits at the intrapopulation level

The study was conducted under permits (licence number 261/2023/CAPT) of the Portuguese governmental administration and conformed to international guidelines and national legislation regarding animal capture, manipulation, and experimentation for scientific purposes.

To test possible intrapopulation differences in biological traits between individuals from the core and the front of the invasion gradient in the Rabaçal River, crayfish abundance, total length, sex-ratio, body condition, behavior, and trophic niche were evaluated. Crayfish were captured by placing 8 and 12 funnel traps in the core and front sites (Fig. 1), respectively, for 24 h. These traps were specifically used for decapod crustaceans, they were all cylindrical (43 cm *d*, 22 cm *h*; 1.5 cm mesh) and were baited with dead fish (*Trachurus trachurus*). The captured individuals were counted, weighted, and measured from the rostrum tip to the telson rear edge (total length), and sex was determined following Sousa et al. (2013). The abundance of the signal crayfish per site was expressed as the total number of individuals per trap for 24 h (catch per unit of effort, hereafter ind. CPUE). In addition, the body condition of signal crayfish was assessed using individuals randomly collected in the field. A total of 80 (51 females and 29 males) and 38 (18 females and 20 males) individuals without visible injuries with a total length between 8 and 11 cm (in order to reduce possible bias; Rebrina et al. 2015), were measured in the core and front sites, respectively. For this, Fulton's condition factor was used following Rebrina et al. (2015), and it was calculated with the equation $K_c = 100 \cdot W/L^3$, where *W* is the total weight (g) of signal crayfish and *L* is the total length (cm).

Assessment of behavioral differences at the intrapopulation level

For the behavioral tests, a total of 40 captured signal crayfish in the field were immediately transferred to separate individual aquariums (to prevent any interaction between individuals) without food, located at the Polytechnic Institute of Bragança, where they remained for three days at a water temperature similar to field summer conditions (21 °C).

Two tests were conducted, each one using unique sets of 16 randomly selected individuals (8 females and 8 males) from both the core and the front of the invasion gradient (Fig. 1). The decision to include both males and females in our experiments was based on previous studies that have shown that sex can significantly influence crayfish behavior (Nakata and Goshima 2003; Gherardi and Cioni 2004). The experimental assessments were performed in an environmentally controlled room set at 21 °C in an aquarium with 46 × 63 × 32 cm (Suppl. material 1: fig. S1), and each individual was only used once. All individuals used in the behavioral tests were carefully examined to ensure they were intermoult and free of visible injuries in order to reduce possible bias (following Hudina et al. 2011).

The aim of the first and second tests were to evaluate boldness behavior associated with the presence of food and a novel environment, respectively. Average (\pm SD) of the signal crayfish from the core and front sites were 9.3 (\pm 0.6) cm and 9.6 (\pm 0.6) cm in the first test and 9.0 (\pm 0.7) cm and 8.9 (\pm 0.9) cm in the second test. Before testing, crayfish were allowed a 5-minute acclimatization period within a shelter (17 \times 20 \times 16 cm) placed inside the main aquarium, with the tested stimulus (food or the novel environment) already present in the main aquarium (following Brown et al. 2007; Suppl. material 1: fig. S1). After this period, the shelter lid was removed to initiate the tests. In the first test, a food source (cat food) positioned 30 cm away from the shelter at the opposite end of the aquarium was present. In the second test, a deep box open from above filled with stones and freshwater mussel shells was positioned 20 cm away from the shelter to simulate a novel environment (Suppl. material 1: fig. S1). In both tests, the time taken to exit the shelter was measured and the individual was considered to have left the shelter when its entire body was outside. New aquariums were used in each individual test. In both tests, the experiment was finished if the tested individual did not exit the shelter in 15 minutes.

Assessment of trophic niche at the intrapopulation level

To explore the trophic niche between the core and front of the invasion gradient, a total of 15 males and 15 females in the intermolt stage and without signs of injuries were randomly collected in both core and front sites of the invasion gradient (Fig. 1). The animals were measured (total length varied between 6.5 and 10.5 cm in the core and between 7.0 and 11.8 cm in the front) and immediately killed by freezing and were kept at -20 °C until the day of analysis. For stable isotope analysis, the animals were thawed; the abdominal muscle was extracted, dried in an oven for 24 hours at 40 °C, and then homogenised using a ceramic mortar. The elemental and isotopic composition analyses were conducted using a Thermo Scientific Flash 2000 model Organic Elemental Analyzer (EA), linked to a Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS) via ConFlo IV. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of the samples and standards underwent normalization with certified reference materials for each element—IAEA-N-1, IAEA-N-2, and IAEA-NO3 for nitrogen, and USGS-24 and USGS-40 for carbon, achieving an analytical error margin of about 0.1‰. The isotopic composition of animal tissue samples was determined with precision, using an internal standard of sea bass, which was read after every 12 analyses to ensure analytical control. Corrections for the mass effect on the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values were made according to a calibration curve derived from the delta values of N and C, which in turn were based on the peak amplitudes of various masses within the caffeine standard. Furthermore, the elemental composition was ascertained utilizing the K factor of *Chlorella*, with samples being analyzed in duplicates to maintain a coefficient of variation below 10%.

Assessment of ecological effects at the intrapopulation level

Individuals of *Physella acuta* (Draparnaud, 1805) were used to assess consumption rates of crayfish individuals from the core and front of the invasion gradient. The individuals of *P. acuta* were captured using a hand net in the Fervença River.

We used this non-native species to reduce possible ethical problems associated with high prey mortality (see results) and because gastropod species are usually a preferred food item for signal crayfish (Rosewarne et al. 2016). The collected animals were rapidly transported to the laboratory and the shell length of individuals was measured from the apex to the tip of the last whorl with a caliper. The shell length varied between 6 and 10 mm, with an average (\pm SD) of 6.97 (\pm 1.12) mm. A total of 70 (35 individuals from the core and 35 from the front; Fig. 1) intermoult male signal crayfish captured in the Rabaçal River without signs of injuries were also used in this experiment. These signal crayfish captured in the field were immediately transferred to individual aquariums (to prevent any interaction between individuals), located at the Polytechnic Institute of Bragança, where they remained without food for three days at a water temperature similar to field conditions (21 °C). The selected individuals had an average length (\pm SD) of 8.2 (\pm 0.5) cm and 8.3 (\pm 0.5) cm in the core and front of the invasion gradient, respectively.

A total of 7 treatments with different abundances (2; 4; 8; 16; 32; 64; and 128 snails) of *P. acuta* were prepared. For each treatment, 5 replicates were used. Then, individuals of *P. acuta* were introduced into aerated aquariums (40 × 20 × 20 cm) containing 10 L of water but no sediment. Subsequently, one male signal crayfish individual was introduced into each aquarium. The signal crayfish individuals remained inside the aquariums for 24 h at a room temperature of 21 °C, and at the end of this period, the number of *P. acuta* individuals consumed were determined. The control treatment consisted of aquariums with *P. acuta* using the same abundances as described above but without crayfish.

Another laboratory experiment was conducted to assess possible differences in leaf mass loss following Carvalho et al. (2018, 2022). In summary, a total of 18 aquariums (40 × 20 × 20 cm) were prepared to replicate the most natural environment possible for the crayfish, so that their behavior was changed as little as possible. They were placed in a room with a temperature of 21 °C with an individual aeration system and, in each aquarium, stones were placed to provide a hiding refuge for the crayfish. Three different treatments were considered: six control aquariums with no crayfish; six representing the core of the invasion gradient; and the other six representing the front (Fig. 1) of the invasion gradient. A total of 10 L of water were added to each aquarium. After 24 h of aeration, the crayfish were placed in the aquariums and left 24 h without food. We only used male crayfish of approximately the same total length (average of 7.9 (\pm 0.5) cm and 8.0 (\pm 0.6) cm in the core and front treatments, respectively). Mesh bags with 4 g of dry alder leaves *Alnus lusitanica* were also prepared. Several other leaf-containing mesh bags were previously placed in the Rabaçal River for one week to be colonized by local microbiota (see Carvalho et al. 2018 and 2022 for detailed methodology); these were placed next to the prepared 4 g food bags for 24 h to serve as inoculum. After this, the leaves were removed from the 4 g food bags and placed in the aquariums for a period of 17 days (following Carvalho et al. 2018 and 2022). This time-period was more than sufficient (see results) to see a meaningful consumption of leaves. At the end of the experiment, the remaining leaves were collected with the aid of a 500 μ m sieve and then placed in an oven at 60 °C for 48 h. Finally, the leaves were weighed and the difference between the initial (4 g) and the final weight for each aquarium was calculated and then converted into a percentage of leaf mass loss. At the end of the

experiment, two water samples of 50 ml from each aquarium were collected to assess and compare the concentration of dissolved nutrients (ammonia, nitrite, nitrate, and phosphate) between the control, core and front treatments. For each nutrient, specific protocols were followed, using colorimetric methods (for details see Grasshoff et al. 1999). The readings were taken using a spectrophotometer HACH DR/2000 (HACH, Loveland, CO).

Data analysis

Differences in abundance between sites were analysed by non-parametric Kruskal-Wallis's tests, since the analysed datasets did not meet homoscedasticity and/or normality assumptions, even after several transformations. Differences in crayfish total body length and body condition between sites, sex and their interaction were compared by parametric two-way ANOVA. A chi-squared goodness-of-fit test was used to compare the observed numbers of males and females in the core and front sites with the numbers expected under a sex ratio of 1:1.

A two-way ANOVA was performed to investigate potential differences in stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) between sites, sex and their interaction. Following this, the isotopic niches of signal crayfish individuals from the core and front sites were analyzed using the SIBER method (Stable Isotope Bayesian Ellipses in R), as introduced by Jackson et al. (2011). This method entails the computation of ellipse areas, which were adjusted using SEAc (Standard Ellipse Area Correction) to account for sample size and other biases. The ellipses were then extended using Bayesian modelling to generate the SEAb (Bayesian Standard Ellipse Area). This approach allows for the comparison of isotopic niche sizes between groups. The application of SEAb served as a measure for comparing the relative sizes of ecological niches within different groups (Jackson et al. 2011). This was determined by the dimensions of the ellipses that were modelled using the isotope data, and by their predicted posterior distributions. When two groups have similar SEAb values, it suggests that the width of their isotopic niches is comparable, which indicates that their dietary ranges are likely similar (Jackson et al. 2011).

A two-way ANOVA was also performed to investigate potential differences in the time taken to exit the shelter for food or a new environment between sites, sex and their interaction. In addition, a Z-test was used to compare the proportions of individuals from front and core sites that exited the shelter within 15 minutes, to determine whether the observed difference was large enough to reject the null hypothesis that there was no difference between the two groups.

Two-way ANOVA was performed to evaluate gastropod consumption between individuals from the core and the front of the invasion gradient in the different abundance levels. One-way ANOVA was used to compare leaf mass loss and nutrients between treatments in the laboratory experiment.

When necessary, analyses were preceded by Shapiro-Wilk test to check the normality of the residuals and the Bartlett test to check for homoscedasticity, or normality was assumed if the number of observations satisfied the assumptions of the central limit theorem (Zar 1999). In the one and two-way ANOVAs we used Tukey "post hoc" tests to assess possible differences between pairs of groups. All the statistical analyses were carried out using the packages "SIBER" "ggplot2", "stats", "cowplot", "pgirmess", "phia" and "multcomp" (R Core Team 2021).

Results

Environmental characterization

The sampled sites are separated by 15 km and have very similar environmental conditions (Suppl. material 1: table S1). The studied river is subjected to a very low human disturbance and the RHS survey gives very similar results for both sites (Suppl. material 1: table S1). Consequently, we assume that the observed differences in biological traits and ecological impacts (see below) are not related to possible distinct environmental conditions between core and front sampling sites.

Biological traits at the intrapopulation level

Significant differences in abundance were found between the two sites (Kruskal-Wallis test, $\chi^2 = 8.15$, $p = 0.004$), with the core of the invasion gradient showing higher abundance (17.25 ± 13.38 ind. CPUE) when compared to the front (4.38 ± 4.47 ind. CPUE) (Fig. 2A).

Significant differences in the crayfish body length were found between sites (two-way ANOVA, $F = 132.32$, $p < 0.001$). However, there was no significant effect of sex ($F = 2.11$, $p = 0.15$) nor a significant interaction between site and sex ($F = 0.45$, $p = 0.50$). Signal crayfish individuals in the front of the invasion gradient were significantly larger than those in the core (Fig. 2B).

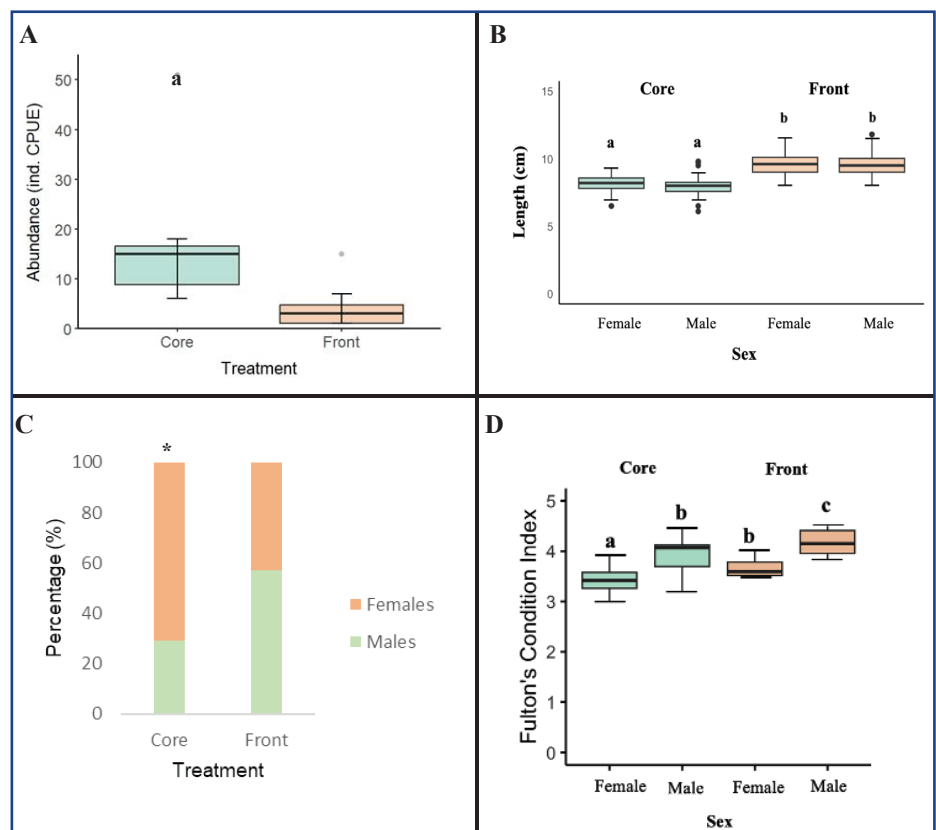


Figure 2. Abundance (A), body length (B), sex ratio (C), and Fulton's body condition index (D) for signal crayfish (*Pacifastacus leniusculus*) in the core and front of the invasion gradient. Boxplots show median values (central line), the interquartile range (box), and the non-outlier range (whiskers), and dots represent extreme values. Different lowercase letters or asterisk indicate significant differences.

There was a significant deviation from expected 1:1 sex ratio in the core of the invasion gradient ($\chi^2 = 24.38$, $p < 0.001$), which was dominated by females (71%). Males were slightly more numerous at the front of the invasion gradient (57%), but this was not significantly different from equal sex ratio ($\chi^2 = 0.714$, $p = 0.40$) (Fig. 2C).

Significant differences in the signal crayfish body condition using the Fulton's Condition Index were found between sites (two-way ANOVA, $F = 37.85$, $p < 0.001$), sexes ($F = 94.39$, $p < 0.001$), but no significant differences in the interaction between site and sex were detected ($F = 0.02$, $p = 0.89$), with individuals from the front of the invasion gradient and males presenting higher values (Fig. 2D).

Behavioral differences at the intrapopulation level

We observed no significant differences in the time for exiting the shelter with food between sites (two-way ANOVA, $F = 0.12$, $p\text{-value} = 0.731$), sexes (two-way ANOVA, $F = 0.01$, $df = 1$, $p\text{-value} = 0.96$) or the interaction between site and sex (two-way ANOVA, $F = 0.01$, $p\text{-value} = 0.95$). Similarly, we observed no significant differences in the time for exiting the shelter for new environment between sites (two-way ANOVA, $F = 0.742$, $p\text{-value} = 0.396$), sexes ($F = 2.34$, $p\text{-value} = 0.14$) or the interaction between site and sex ($F = 2.89$, $p\text{-value} = 0.10$).

However, there was a significant difference in the proportion of individuals that exited the shelter between the core and front sites (Z-test, $p = 0.032$). A higher proportion of individuals exited the shelter at the front site. Notably, 8 out of 16 individuals from the core group but only 2 out of 16 from the front group remained in the shelter.

Trophic niche at the intrapopulation level

Significant differences between individuals from the core and front of the invasion gradient was observed for $\delta^{15}\text{N}$ (two-way ANOVA, $F = 11.07$, $p = 0.002$) and $\delta^{13}\text{C}$ ($F = 10.51$, $p = 0.002$) values. In contrast, no significant differences were found between sex for either $\delta^{15}\text{N}$ ($F = 0.86$, $p = 0.36$) or $\delta^{13}\text{C}$ ($F = 1.78$, $p = 0.19$) values and interaction between site and sex for $\delta^{15}\text{N}$ ($F = 1.15$, $p = 0.29$) or $\delta^{13}\text{C}$ ($F = 0.53$, $p = 0.47$) values. The Tukey "post hoc" test further confirmed this, showing that the $\delta^{15}\text{N}$ mean value for the core was significantly lower than that for the front of the invasion gradient, with a shift of 0.58‰ (95% CI: -0.58 to -0.15 , $p = 0.001$). On the other hand, the $\delta^{13}\text{C}$ mean value for the core was significantly higher than that for the front of the invasion gradient, with a shift of -0.56‰ (95% CI: -0.92 to -0.21 , $p = 0.002$). The SIBER results highlight a separation between the isotopic niches at the invasion core and front (Fig. 3A). The isotopic niche overlap between males from the core and the front was notably small at 0.03‰^2 , equating to 2% of the area. For females, the overlap was even smaller, at 0.005‰^2 (0.3% of the area). When considering the animals of the same origin, the overlap between sexes was considerable at the front of the invasion gradient for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, at 0.68‰^2 , making up 54% of the cumulative area. At the core, a moderate male-female overlap was found, corresponding to 0.30‰^2 , or 36% of the area. The areas of the standard ellipses, which correspond to each group, varied, with ranges of 1.25‰^2 and 0.68‰^2 for females and males at the front of the invasion gradient, respectively, and of 0.50‰^2 and 0.66‰^2 for females and males at the core of the invasion gradient, respectively (Fig. 3B).

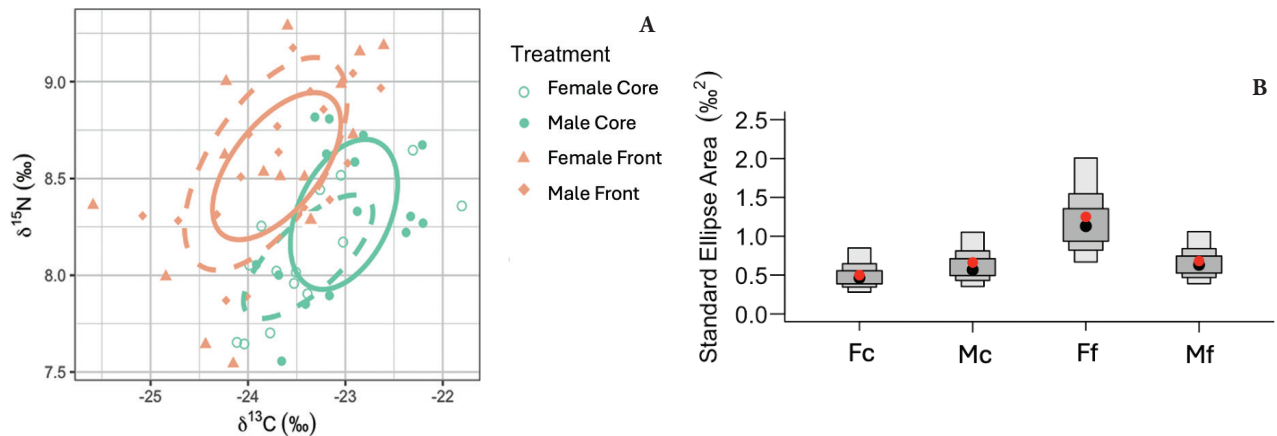


Figure 3. Isotopic niche (A) widths for adult signal crayfish muscle tissue from the core (blue) and front (red) individuals, with their sample-size corrected standard ellipses (SEAc). Standard ellipse areas (SEAb) (B) for the core: female (Fc) and male (Mc); and front: female (Ff) and male (Mf). Dashed and solid ellipses represent females and males from both the core and the front of the invasion gradient, respectively. The boxes represent the 95, 75, and 50% credible intervals, with the mode indicated by a black dot. The maximum likelihood estimate for the corresponding SEAc is indicated by a red dot.

Ecological impacts at the intrapopulation level

No gastropods were found dead in the control treatment. When analyzing *P. leniusculus* gastropod consumption, there was a significant interaction between abundance level of snails and the origin of the signal crayfish ($F = 8.51$, $p < 0.001$). In mesocosms with 2, 4, 8, 16, and 32 *P. acuta* individuals, practically all of them were consumed by the signal crayfish with no significant differences between individuals from the core and front of the invasion gradient (Fig. 4A, Suppl. material 1: table S2). However, for abundance levels of 64 and 128, significantly more gastropods were consumed by crayfish from the front of the invasion gradient ($\chi^2 = 18.15$, $p < 0.001$; $\chi^2 = 52.74$, $p < 0.001$, respectively) (Fig. 4A, Suppl. material 1: table S2).

Significant differences in the percentage of leaf mass loss were also detected (one-way ANOVA, $F = 70.30$, $p < 0.001$), with individuals from the front of the invasion gradient consuming more leaves (Fig. 4B). Both front and core groups were significantly different from the control treatment (Tukey "post hoc" test, $p < 0.001$ and $p < 0.01$, respectively), as well as between each other (Tukey "post hoc" test, $p < 0.001$).

Significant differences were detected for nitrate concentrations in the water after the leaf consumption experiment (one-way ANOVA, $F = 4.90$, $p = 0.023$) (Suppl. material 1: table S3), with the highest values in the treatments with signal crayfish from the front site (Fig. 5A). Significant differences were detected between front and control (Tukey "post hoc" test, $p = 0.048$) (Suppl. material 1: table S4) and between core and front treatments (Tukey "post hoc" test, $p = 0.034$) (Suppl. material 1: table S4). Significant differences were also detected for phosphate concentrations (One-way ANOVA, $F = 8.15$, $p = 0.004$) (Suppl. material 1: table S3), with the highest values in the front treatment (Fig. 5B). However, while significant differences were detected between front and control treatments (Tukey "post hoc" test, $p = 0.002$) (Suppl. material 1: table S4) the differences between the core and front treatments were not significant (Tukey "post hoc" test, $p = 0.069$) (Suppl. material 1: table S4). No significant differences between treatments were found for ammonia (One-way ANOVA, $F = 1.06$, $p = 0.375$) (Suppl. material 1: table S3, Fig. 5C) or for nitrite (One-way ANOVA, $F = 2.07$, $p = 0.172$) (Suppl. material 1: table S3, Fig. 5D) concentrations.

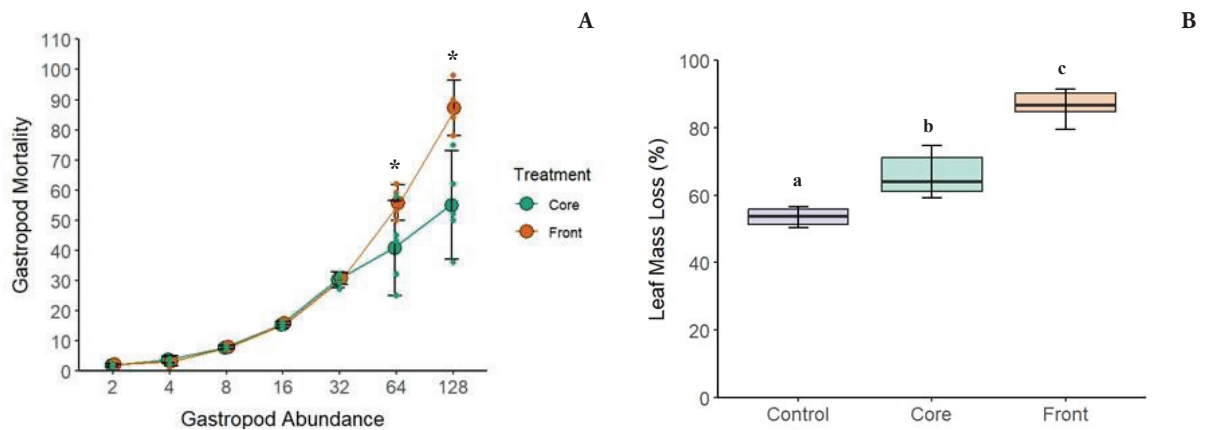


Figure 4. Number of gastropods consumed by the signal crayfish (*Pacifastacus leniusculus*) in each abundance level for core and front treatments (A) and leaf mass loss (%) for control, core and front treatments (B). The circles in A represent the mean mortality values in each prey abundance level for the core (in red) and front (in blue) treatments; smaller red and blue dots represent the number of consumed gastropods in individual replicates. Boxplots in B show median values (central line), interquartile range (box), and range (whiskers). Different lowercase letters or asterisks indicate significant differences.

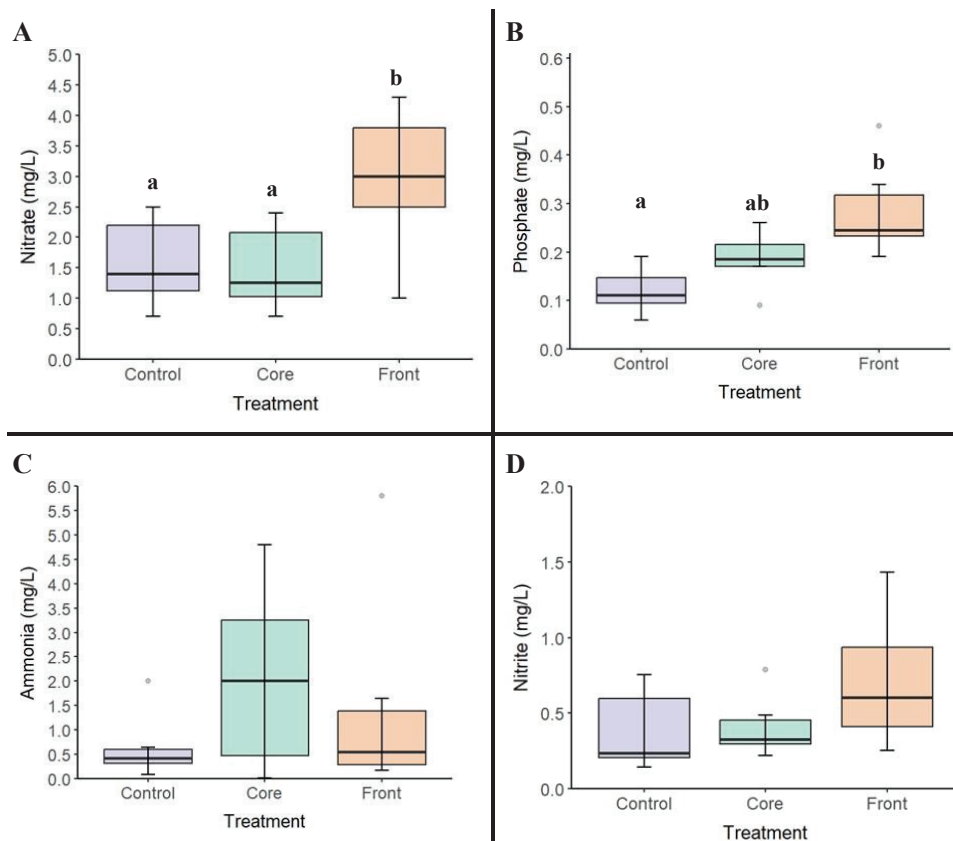


Figure 5. Concentrations (mg/L) of nitrate (A), phosphate (B), ammonia (C) and nitrite (D) in control mesocosms and in mesocosms with individuals from the core and front of the invasion gradient. Boxplots show median values (central line), interquartile range (box), range (whiskers), and dots represent extreme values. Different lowercase letters indicate significant differences.

Discussion

Natural populations consist of phenotypically diverse individuals who exhibit variation in their biological traits (Bolnick et al. 2011). However, these differences are usually neglected in biological invasion studies and most work is devoted to assessing ecological impacts at the species level (Haubrock et al. 2024). In this study,

we highlight that several biological traits and ecological impacts may vary along an invasion gradient with clear differences established at the intrapopulation level. In the particular case of the signal crayfish in the Rabaçal River, we clearly showed that abundance was higher in the core of the invasion gradient and females dominated there; while larger individuals with a higher body condition were detected at the front of the invasion gradient where slightly more males than females were captured. Regarding behaviour, we demonstrated that the animals in the front were bolder. In the same vein, individuals from the core and front of the invasion gradient occupied clearly distinct trophic niches, with individuals from the front having higher $\delta^{15}\text{N}$ and lower $\delta^{13}\text{C}$ values. These differences in biological traits are consistent with distinct ecological impacts as assessed by aquarium experiments, with higher consumption of gastropods, leaf mass loss, and release of nutrients by individuals from the front of the invasion gradient.

Biological traits at the intrapopulation level

Signal crayfish abundance was lower in the front compared to the core of the invasion gradient and individuals were larger at the front of the invasion. On a simplistic level, it is obvious that in the newly invaded sites, there will be lower abundance, given that signal crayfish individuals are still arriving at the front of the invasion. Similar differences in abundance and size were also reported in many other animal invasions (see for example Bøhn et al. 2004; Wolf and Weissing 2012; Brandner et al. 2013; Raffard et al. 2022), including on non-native crayfish populations (Hudina et al. 2012; Nawa et al. 2024). Differences in size could be explained by the fact that larger individuals can move faster and thus are the ones who lead the invasion (Hudina et al. 2012). Similar findings regarding the influence of size in dispersal were recorded in a study with cane toads, *Bufo marinus*, in Australia, where toads with longer legs were the faster ones and the first to reach new sites and so leading the invasion front (Phillips et al. 2006). Another factor possibly contributing to the differences in size between our study sites could be the lower level of recruitment in the front of the invasion gradient given the low abundance of crayfish in this site. Finally, individuals could be growing faster due to density-dependent effects or due to trophic niche shift to more nutritious food source in the front of the invasion gradient (Hudina et al. 2012; Brandner et al. 2013). In this study, we were not able to determine the main mechanism explaining our results regarding the size of the animals and all these hypotheses should be tested in future works.

Differences in sex ratio were also detected between the core and front of the invasion gradient. At the core, the percentage of females was significantly higher than males but at the front, the percentage of males was slightly higher than females. Higher male percentage in the front of the invasion were also reported for round goby (*Neogobius melanostomus*) in Canada (Gutowsky and Fox 2011), or for other signal crayfish populations in Europe (Capurro et al. 2007; Wutz and Geist 2013). Those results, according to the authors, were related to recent, not fully established invasive populations. The sex ratio is possibly important since it may interfere with some ecological processes. For example, female-biased populations of western mosquitofish *Gambusia affinis*, in which males are substantially smaller than females, are able to induce stronger pelagic trophic cascades than male-biased populations, causing larger impacts on communities and ecosystems (Fryxell et al. 2015). A study on the invasive freshwater crayfish *Procambarus clarkii* showed that females

have larger home ranges (Barbaresi et al. 2004). The higher percentage of females in the core of the invasion might also be related to a higher number of encounters and fights among more aggressive males as a result of higher crayfish abundance and a consequent decrease in available space and resources (Sousa et al. 2013). This might result in the death of some male individuals or the exclusion of the weakest in sites with higher abundance. Alternatively, it can be related to bolder male crayfish individuals that will be more prone to explore downstream areas (Raffard et al. 2022).

We also observed that signal crayfish individuals in the front of the invasion gradient had a higher body condition when compared with the individuals in the core. This may be related to a lower competition for resources at the front, which allows these individuals to have easier access to a higher amount or more nutritious food (see below further discussion on trophic niche) and to shelters, and in doing so they possibly increase their body condition (Brandner et al. 2013; Rebrina et al. 2015). Although not studied, another explanation for these results may be related to a lower level of predation (e.g. by river otter *Lutra lutra* and brown trout *Salmo trutta*) and/or parasitism in the front when compared to the core. For example, studies with cane toads in Australia showed that pathogens and parasites lag about 2 years behind the front of toad invasion, due to stochastic events that lead to local extinctions or transmission failure of the pathogens/parasites in the front of the invasion (Phillips et al. 2010; Brown et al. 2013). However, this last aspect was not assessed here and future studies should explore this topic. Furthermore, and as described in another study with signal crayfish populations (Rebrina et al. 2015), the condition index is sex-dependent.

Behavioural differences at the intrapopulation level

Several organisms rapidly change their behavioural traits to expand their distribution area and be able to make decisions that involve risks (Yagound et al. 2022) due to the new challenges imposed by the environment and the pressures arising from their expansion (Biro and Stamps 2008). What we observe in this study is a behaviour change in geographical terms, with the animals at the front being bolder and more willing to take risks than those at the core of the invasion gradient (Gruber et al. 2018). Similar results were obtained by Groen et al. (2012) and Myles-Gonzalez et al. (2015) for the round goby in non-native areas where the individuals at the front took more risks and moved faster. Several studies have also concluded that individuals at the front of the invasion gradient tend to be more exploratory than those at the core (Atwell et al. 2012; Liebl and Martin 2012, 2014).

Trophic niche at the intrapopulation level

In addition to differences in several biological traits as discussed above, we also observed higher $\delta^{15}\text{N}$ and lower $\delta^{13}\text{C}$ values in signal crayfish individuals from the front compared to the core of the invasion gradient. These differences in $\delta^{13}\text{C}$ suggest a greater reliance on submerged vegetation, periphyton, and detritus as important dietary sources in the core, possibly reflecting the increased competition for resources in this site (Ercoli et al. 2021). The elevated $\delta^{15}\text{N}$ values observed in signal crayfish at the invasion front suggest a dietary shift towards a reliance on a higher proportion of consumers such as macroinvertebrates. This shift in diet is likely a response to the specific ecological conditions at the invasion front, where competition for

resources is presumably less intense compared to the core of the invasion gradient, where crayfish abundance is significantly higher (Brandner et al. 2013; Hudina et al. 2017). Although direct diet sources were not assessed in this study, it is plausible that crayfish in core areas consume more submerged vegetation and detritus. Notably, female crayfish in the core of the invasion gradient exhibited a smaller niche area, suggesting that the heightened competition for limited food resources compels them to consume a lower range of dietary items at lower trophic levels. In contrast, signal crayfish at the front of the invasion gradient may have greater access to invertebrates and other animal prey (possibly including dead animals), which are readily available in the initial phase following their arrival. The relatively low abundance of crayfish at the front of the invasion gradient likely reduces competition, facilitating this reliance on higher trophic levels (Bubb et al. 2004; Brandner et al. 2013; Greenhalgh et al. 2022). Increased boldness among crayfish at the front of the invasion gradient could further enhance their ability to exploit these available resources, thereby providing a competitive advantage in newly invaded areas. However, this situation remained untested, and future studies are necessary to clarify this situation.

Ecological impacts at the intrapopulation level

This study also highlighted that the ecological impacts may vary along the invasion gradient with clear differences established at the intrapopulation level. In the particular case of the signal crayfish in the Rabaçal River, the consumption of gastropods, leaf mass loss, and the concentration of nitrates and phosphates were significantly higher in the aquariums with crayfish individuals from the front of the invasion gradient.

Dispersal is one fundamental ecological process where these intraspecific variations, particularly the personality traits (e.g. activity, boldness, aggression, and exploration) may play a crucial role (Daniels and Kemp 2022). Personality traits are often correlated with dispersal and this correlation is also known as a dispersal syndrome (Biro and Stamps 2008; Galib et al. 2022; Raffard et al. 2022). These dispersal syndromes have been reported in a wide range of taxa such as great tits, *Parus major* (Dingemanse et al. 2003); common lizards, *Lacerta vivipara* (Cote and Clobert 2007); North American red squirrels, *Tamiasciurus hudsonicus* (Cooper et al. 2017); mud crabs, *Panopeus herbstii* (Belgrad and Griffen 2018), among others. Activity, boldness, and exploration traits are often linked with better fitness, and individuals with these traits enhanced are expected to disperse further (Juette et al. 2014; Galib et al. 2022), and in so doing they lead the invasion front. Some of these personality traits have previously been observed in the signal crayfish (Galib et al. 2022), and our results also showed that signal crayfish individuals in the front of the invasion gradient were bolder than individuals in the core.

Since individuals with this type of behaviour are more prone to disperse, this can be associated with higher energy expenditure, which was counterbalanced by higher consumption rates (Raffard et al. 2022). This could be the main reason explaining our results regarding gastropod consumption, where we observed no differences between core and front individuals in the low prey abundance treatments but higher consumption of prey in the high-abundance treatments by the crayfish from the front of the invasion gradient. Similar results were also obtained in the experiment with leaf mass loss. This suggests a higher voracity of crayfish from the front of the invasion gradient because of their higher metabolic demands (Raffard et al. 2022).

Our study also made it possible to verify that the signal crayfish influences key ecosystem processes, as is the case of leaf litter processing and nutrient cycling. We observed that signal crayfish had an effect in leaf litter processing. Similar results have been reported for the red swamp crayfish, *Procambarus clarkii* (Carvalho et al. 2016 and 2018). Since leaf litter processing is driven by a group of animals that control the flux of carbon and energy in aquatic food webs (Carvalho et al. 2018), fundamental processes like nutrient cycling and organic matter turnover (Jackson et al. 2014), could be affected by the invasion of signal crayfish, even at the intrapopulation level. In addition, higher nutrient concentrations were also found in the front treatment, in line with a study by Villéger et al. (2012) where *Salmo trutta* individuals that were capable of dispersing further were the ones that displayed a higher excretion rate of nitrogen and phosphorus.

Overall, we found a higher consumption of gastropods and leaf litter, and a higher concentration of nutrients, namely nitrates and phosphates, in the experiments with crayfish from the front of the invasion gradient, where bigger and bolder individuals, supposedly with higher metabolic rates, were present. Therefore, these intrapopulation interactions can cascade to the population and community levels due to a different consumption of prey and/or leaf litter and being responsible for nutrient-mediated effects, modifying nutrient cycling at the ecosystem level. However, and as clearly demonstrated here, although the per capita consumption of prey and excretion rates were significantly higher at the front of the invasion gradient it should be noted that the abundance in the two sites is quite different, being much higher in the core of the invasion gradient.

The results of this study clearly demonstrated significant differences at the intrapopulation level, but some questions still remain unanswered and open the door for future studies. For example, it would be interesting to assess the metabolic rates in individuals from the core and front of the invasion gradient as the possible key mechanism explaining the different consumption rates and nutrient concentrations reported here. In the same vein, biological traits can also be related to the expression of specific genes (Yagound et al. 2022) and future studies may include a comparison of genetic variation and gene expression between individuals from the core and front of the invasion gradient to test whether the observed changes are due to selection of certain genotypes favouring dispersal or whether the observed differences are due to phenotypic plasticity (and, possibly, differential gene expression) (Cox 2013). Finally, and because animals in the front of the invasion gradient may have left their enemies behind, it would be interesting to assess the level of predation and parasitism in the core and front of the invasion gradient and their possible contribution to the distinct ecological impacts.

Conclusion

In this study, we demonstrated distinct biological traits and ecological impacts of signal crayfish along an invasion gradient. Currently, theoretical and empirical studies in biological invasions focus mainly on the species level (e.g. compilation of deny lists), especially those that have high ecological and economic impacts. However, as shown here, biological traits and ecological impacts can be very different at the intrapopulation level. Recognizing that individual variation has important ecological and evolutionary consequences, the assessment of biological traits at the intrapopulation level may help better predict the success of dispersal and the

ecological impacts generated by non-native species, with eventual pay-offs in the implementation of meaningful management actions. In the particular case of this study, it could be interesting to implement targeted removal and population control in the front of the invasion gradient to reduce density-dependent processes and remove disperser-adapted phenotypes from the gene pool.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: RS. Formal analysis: HA; DG; ABN; AT; JD; RS. Funding acquisition: RS. Investigation: HA; DG; ABN; AT; JD; RS. Methodology: RS. Resources: RS. Supervision: RS. Writing – original draft: HA; DG; RS.

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Data availability

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

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Supplementary material 1

Additional information

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Data type: docx

Explanation note: **fig. S1.** Experimental setup for testing signal crayfish behavior. (a) The shelter and food were placed at opposite ends of the aquarium. (b) The shelter with a new environment consisting of stones and shells of freshwater mussels. **table S1.** Environmental characterization of Core and Front sampling sites. HMS - Habitat Modification Score; HQA - Habitat Quality Assessment. **table S2.** Summary of Chi-square (χ^2) tests for each abundance level of gastropods. The asterisk show a significant difference in abundance levels between the core and front treatments. **table S3.** Summary of One-way ANOVA tests for nutrients concentration. The asterisks indicate significant differences in nutrients concentration between the core, front and control treatments. **table S4.** Summary of Tukey post hoc tests for the concentration of nitrate and phosphate. The asterisks indicate a significant difference in concentration of nitrate and phosphate between the core, front and control treatments.

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