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Research Article

Mangrove-associated Pacific oysters (*Magallana gigas*) influence estuarine biodiversity

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Abstract

Estuarine ecosystems are often characterised by endemic foundation organisms which facilitate ecosystem interactions and processes established over millennia. Introduction of non-native foundation species can significantly alter ecological communities and functions. Here, we assessed the effects of introduced, reef-forming Pacific oyster *Magallana gigas*, within a temperate Australian mangrove-dominated estuary. Specifically, we investigated whether mangrove-attached *M. gigas* oyster reefs influenced intertidal fish and invertebrate communities, and fish foraging behaviour. We measured and contrasted the benthic structure and faunal communities of fringing bare sediment, *Avicennia marina* mangrove and Pacific oyster *M. gigas* reef habitats using a combination of quadrats, fyke nets and remote unbaited video (RUV) surveys. Pacific oyster reefs showed no impacts on pneumatophore density or morphology, but were positively associated with higher seedling densities. Furthermore, invertebrate and fish community metrics (e.g. richness, biomass, length) were typically higher in *M. gigas* reefs compared to the other habitats. However, several mobile, non-native species were predominantly detected in *M. gigas* reefs, including exotic gobies and European shore crabs *Carcinus maenas*, a declared marine pest. Overall, we found that *M. gigas* reefs in fringing mangrove forests were associated with biodiverse faunal communities, including fisheries-targeted species, but also facilitated other non-native species. These outcomes highlight some of the ecological pros and cons of non-native oyster reefs and the complexity of managing estuaries globally where Pacific oysters increasingly co-occur with endemic habitat-forming species.

Key words: Biogenic, facilitation, habitat-formers, invasive species, mangroves, nekton, shellfish, temperate

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Introduction

In estuarine systems, habitat-forming, foundation species including mangroves, seagrass, oysters and coral, influence community composition and ecosystem functioning (Dayton 1972; Angelini et al. 2011). Habitat-formers directly modify the physical structure and biogeochemical processes and indirectly influence the environment from the activities of other organisms they facilitate. Subsequently, habitat-formers support valuable ecological services including fish production, nutrient cycling and coastal protection (Barbier et al. 2011; zu Ermgassen et al. 2021). For example, mangroves protect coastal infrastructure and stabilise otherwise bare coastal margins (Marois and Mitsch 2015). Globally, mangrove forests also store an estimated 5.85 Pg of carbon (Alongi 2020). Similarly, oyster reefs remove excess nitrogen via biofiltration (Ray and Fulweiler 2021) and are used as nursery habitats by fisheries-targeted nekton (Gilby et al. 2018; zu Ermgassen et al. 2021). Positive ecological functions can be further enhanced by multiple, co-existing habitat-formers, which interact to increase structural and functional heterogeneity, leading to facilitation cascades that enhance niche availability and species biodiversity (Thomsen et al. 2022). As an example of a facilitation cascade, intertidal bivalve recruitment and survival may be enhanced by the shading of saltmarsh plants, which in turn, benefit from greater sediment stabilisation and enrichment provided by the bivalves (Gagnon et al. 2020), with their co-occurrence facilitating enhanced invertebrate biodiversity (Angelini et al. 2015). Conversely, when a habitat-former is introduced outside its native range, it can have mixed and context-specific outcomes, including "negative" impacts, such as declines in endemic biodiversity, ecosystem functions or services; or "positive" impacts, the opposite (Guy-Haim et al. 2018; Rilov et al. 2024).

Invasions by non-native species can significantly modify ecological communities and functions, often decreasing species abundance and diversity via negative interactions including competition, predation and altering environmental conditions (Gallardo et al. 2016). Non-native habitat-formers, specifically, can have ecosystem-scale impacts via habitat alteration (Rilov et al. 2024). For instance, the non-native seagrass *Halophilia stipulacea* has displaced native seagrasses in the Caribbean, altering invertebrate communities and limiting nutrient availability (Muthukrishnan et al. 2020). Conversely, non-native habitat-formers may enhance biodiversity, including native (Rodriguez 2006) and other non-native species (i.e. 'secondary invasions', O'Loughlin and Green (2017)). For example, in northern Europe, Pacific oyster *Magallana gigas* (formerly *Crassostrea*) reefs support valuable ecosystem services including nutrient cycling (Zwerschke et al. 2019), shoreline protection (De Vriend et al. 2014) and enhanced shorebird foraging opportunities (Herbert et al. 2018). Zwerschke et al. (2019) proposed that non-native *M. gigas* reefs may restore the ecological functions of almost extinct native oysters *Ostrea edulis* reefs, with McAfee and Connell (2021) suggesting that the global spread of this invader potentially compensates for the worldwide loss of > 85% of endemic oyster reefs (Beck et al. 2011).

Pacific oysters, endemic to the north-west Pacific, have been introduced deliberately for aquaculture and unintentionally through shipping and are now globally distributed on every continent, except Antarctica (McAfee and Connell 2021). They are favoured in aquaculture because of their fast growth rate, high reproductive output and relatively low disease mortality (Troost 2010; Stechele et al. 2022). These traits have also supported invasive *M. gigas* populations establishing in 36 countries, as reviewed by Martínez-García et al. (2022). In the future, *M. gigas* may also spread to dominate most temperate coastlines (McAfee and Connell 2021), with projected climate change conditions favourable to their poleward expansion (King et al. 2021). The proliferation of invasive *M. gigas* populations has created a dilemma for coastal management between encouraging non-native oyster production and restorative aquaculture (van der Schatte Olivier et al. 2020; Theuerkauf et al. 2022), versus mitigating the potential socioecological impacts of non-native oyster reefs (Forrest et al. 2009; Herbert et al. 2016).

As summarised in recent literature reviews (e.g. McAfee and Connell (2021); Hansen et al. (2023)), invasions by *M. gigas* are associated with mixed ecological outcomes for endemic communities, which are context- and scale-dependent. For example, Andriana et al. (2020) showed with manipulative plots that Pacific oysters outcompete native blue mussels (*Mytilus edulis*) and displace native habitat-forming seaweeds in the Walden Sea. Work in this same region has also shown that Pacific oysters can influence parasite infections in *M. edulis*, functioning as vectors for disease transmission (Goedknegt et al. 2019). Furthermore, Bazterrica et al. (2022) investigated Pacific oyster reef impacts in Argentinean soft-sediment habitats, identifying that these oyster reefs supported high densities of exotic and cryptogenic invertebrates that were absent from uninvaded cordgrass and bare sediment areas. Conversely, small-scale studies assessing *M. gigas* impacts on invertebrate communities have found comparable assemblages to endemic bivalve ecosystems in northern Europe (Zwerschke et al. 2019) and Australia (Wilkie et al. 2012) suggesting habitat redundancy. Additionally, manipulative subtidal trials in Sweden assessed the habitat value of *M. gigas* versus *M. edulis* shells using drop samplers and found higher abundances and biomass of epibenthic fauna associated with the Pacific oysters (Norling et al. 2015). These examples indicate that the net impacts of non-native *M. gigas* are complex and mediated by a combination of environmental conditions, habitat structural characteristics and interactions with local biological communities.

Pacific oysters were introduced to temperate Australia in the 1940s for oyster aquaculture and they have since established invasive populations, primarily managed as an aquaculture biosecurity risk (Gillies et al. 2018). In Australian estuaries, *M. gigas* may form oyster reefs on consolidated structures (Gillies et al. 2018) and function as secondary habitat-formers attached to grey mangroves *Avicennia marina*, a primary habitat-former (Bishop et al. 2010; Wilkie et al. 2012). This region formerly supported extensive native oyster reefs till the mid-20th century, with estimated losses of > 99% of flat oyster *Ostrea angasi* reefs and > 90% of Sydney rock oyster *Saccostrea glomerata* reefs (Gillies et al. 2020). Subsequently, non-native *M. gigas* reefs may provide an unintended offset to the loss of native oyster reefs in temperate Australia, contrary to contemporary models of habitat decline (Davis et al. 2011; McAfee and Connell 2021).

Despite the growing distribution of non-native oyster reefs worldwide, previous research on their ecological impacts has primarily focused on their impacts to sedimentary communities particularly in Europe (Padilla 2010). Furthermore, few studies have investigated how *M. gigas* reefs influence fish communities (Martin et al. 2024) or their ecological impacts in mangrove forests (Bishop et al. 2010; Gagnon et al. 2020). To address these knowledge gaps, this study investigated fish and invertebrate community composition and habitat occurrence, with comparisons across *M. gigas* oyster reefs and uninvaded bare sediment and *A. marina* mangrove habitats, within a temperate Australian estuary. Specifically, it tested the hypotheses that: (1) *M. gigas* oyster reefs were associated with lower mangrove pneumatophore densities and sizes compared to uninvaded mangroves; (2) greater sediment organic matter percentages from biodeposition, as compared to other habitats; (3) that oyster reefs support greater invertebrate and fish species richness, relative total abundances, combined invertebrate biomass and fish foraging (total bites), compared to other fringing estuarine habitats; and (4) support distinct invertebrate and fish communities to other habitats. This study provides critical insights for evaluating the ecological role of non-native oysters and understanding their interactions with estuarine mangroves, invertebrates and fish communities.

Methods

Site description

The Port River-Barker Inlet Estuary is the largest estuary in Gulf St. Vincent, Australia (34°47'S, 138°31'E; Fig. 1) and the main maritime port for South Australia's capital city of Adelaide since 1837. The Estuary is highly modified from pre-European conditions, with significant declines in coastal vegetation and native shellfish ecosystems due to urbanisation, pollution and channel modifications (Edyvane 1999). Non-native marine species, such as Pacific oysters (*Magallana gigas*), have also become established within the Estuary, with *M. gigas* first detected from intentional, but unauthorised introductions of hatchery stock in 2009 (Wiltshire et al. 2010). Pacific oysters presently occur throughout the Estuary, primarily attached to artificial structures (e.g. rock levees, pylons), grey mangroves (*Avicennia marina*) and bivalve shells.

This study was situated within the Port River-Barker Inlet Estuary along the western shoreline of Torrens Island, which fringes the main Port River channel (Fig. 1). We sampled three habitat types along a two-kilometre low intertidal fringing zone:

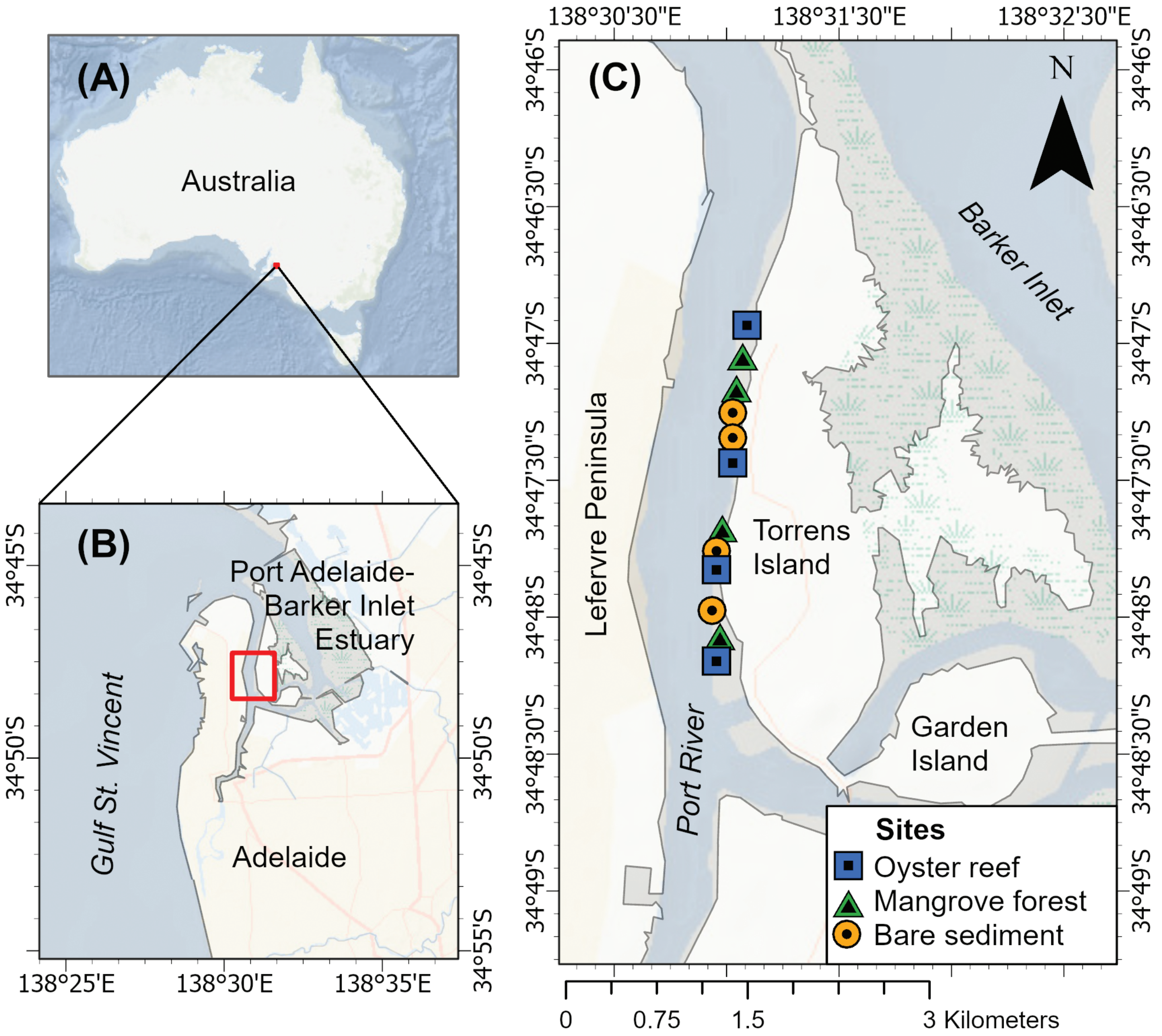

Figure 1. Location of **A** Adelaide in South Australia **B** the Port River-Barker Inlet Estuary and **C** sites of the three surveyed habitat types.

grey mangrove (*Avicennia marina*) forests, used as a natural structured control site, bare sediment and nested *Magallana-Avicennia* oyster beds (hereafter ‘oyster reefs’). Four replicate sites for each habitat (bare sediment, mangroves, oyster reef; Fig. 2) were haphazardly selected (n = 12; Fig. 1). All sites were situated in the mid-intertidal zone above the lowest astronomical tide (LAT) line and were submerged during high tide. Sites were comprised of continuous habitat that extended for ≥ 30 m stretches and separated from other sites by ≥ 30 m. Oyster reefs were comprised of pneumatophore-attached *M. gigas* shell clumps (alive and deceased oysters) which occurred as 50–60 m length patches, extending 5–10 m seawards of fringing mangroves. Reef shell material had a minimum vertical relief of ≥ 50 mm above the surrounding sediment, with typical alive adult (> 25 mm length; Markert (2020)) oyster densities of ≥ 200 ind. m^{-2} (Herbert et al. 2016). Mangrove sites consisted of 50–100 m stretches of fringing old-growth and immature *A. marina* with few or no alive, adult oysters (i.e. < 25 ind. m^{-2}). Bare sediment sites ranged from 30–75 m in length and were characterised by continuous, low-relief habitat of mud/sand and microalgae mats. All site surveys occurred during late February and March 2023.

Quadrat surveys

We characterised site-specific structures during low tide using eight (0.5 × 0.5 m) haphazardly placed photographic quadrats (n = 96; Fig. 2). Photographs were assessed in ImageJ (WS 2011) to estimate benthic cover composition (%) and densities of mangrove seedlings (i.e. < 30 cm height) and pneumatophores. Benthic cover categories were bare sediment, cockle (*Katelysia* spp.) shell, mangrove roots and seedlings and oyster (*Magallana gigas*) shell. Four quadrats per site (n = 48) were excavated to quantify additional structural components and associated macrofauna. Firstly, the aboveground height and basal diameter of up to six haphazardly selected pneumatophores were measured using vernier callipers (to 0.1 mm). Next, we removed all shell material, mobile macrofauna (invertebrates and fish) and the top five centimetres of sediment for processing, including partially buried oyster material (e.g. Grabowski et al. (2005)). Loose material was sieved *in situ* through a 1-millimetre sieve before being bagged, transported to the laboratory and frozen (-20 °C).

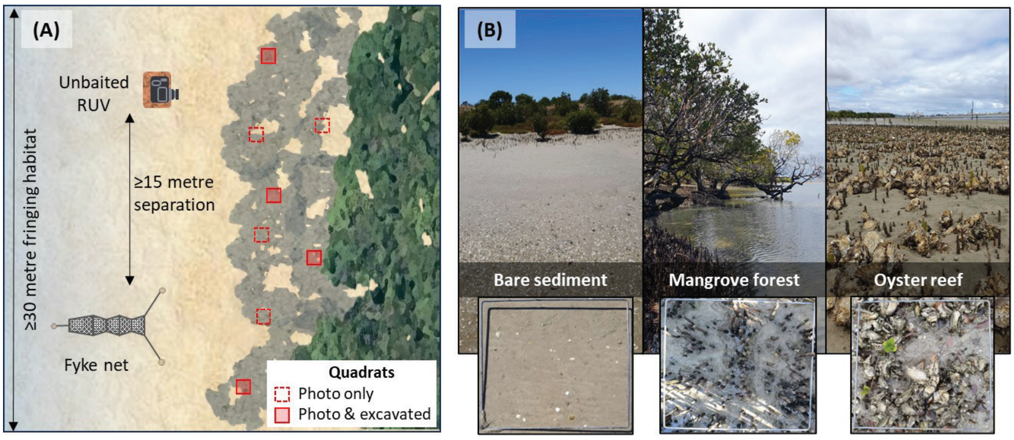

Figure 2. Representative images of **A** simplified survey design used to assess estuarine assemblages at each site, with an unbaited remote underwater video (RUV) system and a fyke net (deployed at high tide) and haphazardly placed quadrats (surveyed at low tide) **B** examples of the surveyed habitats and associated photo-quadrats for bare sediment, mangrove (*Avicennia marina*) forest and mangrove-attached Pacific oyster (*Magallana gigas*) reefs.

In the laboratory, we defrosted, rinsed and sorted the retained material from the excavated quadrats. Firstly, we enumerated oysters and classified them as either alive juveniles (< 2 mm), alive adults (> 25 mm), dead cups (open shell without the top valve) or dead boxes (open shell with two articulated valves). Up to 20 randomly selected alive juvenile and adult oysters per quadrat were measured for shell heights (i.e. longest hinge-lip distance, to 0.1 mm). Additionally, we measured the total biomass oyster and cockle shell material, respectively, using an electronic scale (0.1 g). Total oyster biomass included both the shell and wet meat weights as dead and alive oysters occurred as clumped material, whereas cockle shells consisted of disarticulated and fragmented material. We inspected all shell material for macrofauna, which we enumerated, weighed (wet weight, 0.01 g) and identified to the lowest practical taxonomic level using field guides (e.g. Gowlett-Holmes (2008)).

Nekton surveys

We surveyed nektonic fish (teleosts and elasmobranchs), decapods and cephalopods using a combination of unbaited remote underwater video (RUV) stations and extractive fyke nets (Fig. 2; Martin et al. 2024). We concurrently deployed pairs of RUV and fyke nets from shore across the twelve sample sites over two subsequent days of daylight high tides, with sampling repeated across three sample periods on a fortnightly basis (n = 36). We positioned all survey gear ≥ 15 metres apart during daytime (8:00 to 16:00 h) high tides, in ≈ 1.5 metre depths, for a continuous 90-minutes soak-time.

Each RUV consisted of a single weight-attached GoPro Hero 7/8 camera positioned 20 cm above the benthos. Stations were unbaited to avoid bait-biases influencing habitat use (Bradley et al. 2016). We annotated video records in EventMeasure (SeaGIS 2023) with all nekton counted and identified to the lowest taxonomic level (Gomon et al. 2008). Due to differences in recording duration across RUV surveys, we only annotated the first 60-minutes of each video, commencing 1 minute after deployment to allow disturbed sediment to settle. We calculated the maximum number of individuals of the same species present within a single frame from each video (MaxN), which is a commonly used, conservative abundance metric (Whitmarsh et al. 2016). We also recorded fish foraging behaviour by counting the number of bite interactions observed between fish and the benthos.

We deployed unbaited fyke nets (0.4 m ø opening, fyke mesh size 1.2 mm$^{2;}$ with 2 m x 1.2 m wings; 4 m total length), with the net opening facing the shoreline to sample fish on the ebbing tide (Fig. 2). We removed captured organisms from the net and placed them on to a scaled-grid where we photographed, enumerated, identified them to species level, then released them (except voucher specimens). From each fyke-net deployment, we determined the length measurement of ≤ 20 individuals from voucher specimens and photographs in ImageJ, recorded as either total length (fish), carapace length (decapods) or mantle length (cephalopods) (to 1.0 mm). For any unidentifiable fishes, we euthanised voucher specimens using an overdose of Aqui-S and transported them to the laboratory for identification and measurement.

Abiotic variables

We recorded water temperature (°C) using a multiparameter probe (Polaris C, OxyGuard) and salinity (PPT) using a handheld refractometer prior to each RUV and fyke-net deployment. We estimated distances to estuary mouth from each site using GoogleEarth by measuring the contour along the river edge, as well as distances to the channel edge, representing subtidal refugia (to 1 m). Three replicate sediment samples (≈ 50 g) were collected from each site, transported to the laboratory and immediately frozen. We dried approximately 10 g of defrosted sediment from each sample at 60 °C for at least 4 days, prior to incineration at 450 °C for 4 hours. We then calculated the percentage of sediment organic matter as the difference in weight between pre-combusted and post-combusted sediment.

Statistical analyses

We carried out all statistical analysis in Rstudio (Version 4.3.1; R Core Team 2024). We used non-parametric Kruskal-Wallis chi-squared tests to assess differences in fine-scale habitat components including benthic cover percentages; densities of mangrove structures and shells, shell biomass and sediment organic matter (%). When significant ($p < 0.05$), we used Dunn's post-hoc tests for pairwise comparisons between sites. Mann-Whitney U-tests were used to assess differences in pneumatophore height and diameter between the mangrove and oyster reef sites.

To evaluate the effects of habitat and environmental variables on univariate community metrics (richness, density, biomass) from the quadrats, we first used the Pearson's correlation coefficient to remove collinear predictors ($|r| > 0.7$; Suppl. material 1: fig. S1A). The refined model of environmental predictors included estuary mouth distance, pneumatophore densities, seedling densities and cover (%), cockle shell biomass, oyster shell biomass and sediment organic matter (%). We used Generalised Linear Models (GLMs) to evaluate differences in the univariate invertebrate variables (e.g. species richness, biomass and densities) using the refined model and habitat treatment categories. We assessed species richness using a Poisson distribution, while biomass and density were modelled using negative binomial distributions to meet assumptions of normality and heteroscedasticity. Any significant habitat differences from Analysis of VAriance (ANOVA) results were further assessed using Tukey pairwise comparisons. Modelling was performed using the *PMCMRplus* (v. 1.9.10 Pohlert 2018) and *MASS* packages (v. 7.3-60 Ripley 2011).

Habitat differences in the multivariate community composition from the excavated quadrats was assessed with the *vegan* package (v. 2.6-4, Oksanen et al. 2013) using the PERmutational Multivariate ANalysis of VAriance (PERMANOVA) across Habitats (3 levels, fixed: oyster reef, mangroves and bare sediment) with estuary mouth distance as a covariate. Prior to analysis, we assessed and visualised potential skewness using a series of transformations and shade plots, to achieve a normal distribution. We used fourth-root transformation to reduce the effects of overly abundant invertebrates, before calculating Bray-Curtis dissimilarly matrices. Subsequent post-hoc pairwise PERMANOVA tests assess significant pairwise habitat differences. We then conducted Dufrene-Legendre indicator species analysis using *Labdsv* package (v. 2.1-0, Roberts and Roberts 2016) to identify the species that most contributed to differences between habitat types. We then used a distance-based ReDundancy Analysis (dbRDA) to ordinate the transformed community data and identify relationships with the environmental variables. Using the 'envit' function of the *vegan* package, vectors from the environmental variables were tested for significant ($p < 0.05$) correlation to the ordination axes (9999 permutations) and fitted to the dbRDA plot as overlaid vectors. We also overlaid the Pearson correlations between the indicator species abundances and community data to visualise the strength and direction of associations.

Assessments of nekton communities followed similar steps to those used to assess the assemblages from the excavated quadrats. Firstly, we averaged habitat quadrat variables (e.g. habitat cover %, shell densities) to obtain site-level estimates to use as predictors. Collinear environmental predictors were then removed, based on the Pearson's correlation coefficient ($|r| > 0.7$; Suppl. material 1: fig. S1B). This refined the predictors to estuary mouth distance, percentage cover of *A. marina* seedlings and pneumatophores, cockle shell biomass, oyster shell biomass and water quality parameters (salinity, dissolved oxygen, temperature). Subsequent General Linear Modelling assessed predictors for total abundances and species richness from each survey methods and fish bite counts from the RUV surveys. The GLMs used the refined model of environmental predictors, as well as the factors of Habitat (3 levels) and Sampling Period (3 levels). We assessed species richness using Poisson distributions and used negative binomial distributions for the abundance and count data, based on model requirements.

We investigated potential habitat differences in multivariate nekton assemblages using PERMANOVA tests of Bray-Curtis dissimilarly measures on the square-root transformed data obtained from the RUV and fyke-net surveys, respectively. We added a dummy value to the fyke-net matrices to allow the inclusion of samples containing no nekton. Each PERMANOVA included Habitat (fixed factor; three levels), Sample Period (fixed factor; three levels) and estuary mouth distance as a covariate, with post-hoc pairwise PERMANOVA tests used to examine any habitat differences. We then conducted Dufrene-Legendre indicator species analysis and identified any significantly correlated environmental predicators using 'envfit'. The results were then visualised as distance-based ReDundancy Analysis (dbRDA) plots. Finally, we assessed the size-frequency distribution of all species measured from the fyke nets using Kruskal-Wallis chi-squared tests, with Dunn's post-hoc tests used to assess pairwise differences in nekton species length by habitat type.

Results

Environmental and structural characteristics

Quadrat sampling revealed fine-scale variability within habitats and broader structural differences across habitat types (Table 1, Fig. 2). All benthic habitat cover percentage categories significantly differed across habitats (Table 1; Dunn's Post-hoc: $p \leq 0.004$). Pairwise comparisons supported the proportional dominance of bare sediment cover (%) in bare sediment habitats, as compared to mangrove and oyster reef habitats. Both of these structured habitats had similar percentage cover proportions of cockle shells ($p = 0.33$) and pneumatophores ($p = 0.43$; Table 1). Mangrove and oyster reef habitats also had similar pneumatophore densities ($p = 0.78$) and contained an average of 254 ± 20 pneumatophores m^{-2}.

Oyster reefs had significantly higher proportional cover of oyster (*Magallana gigas*) shells compared to the other habitats, with an average cover of 31.21 ± 2.87 percent (Table 1). This is consistent with the high densities of adult alive oysters (average ± SE: 254 ± 32 ind.m^{-2}) and oyster shell biomass (13.15 ± 1.52 kg.m^{-2}). Cockle (*Katelysia* spp.) shell biomass was also higher in oyster reefs ($p \leq 0.001$), but similar amongst the other habitats ($p = 0.19$). The proportional cover and densities of mangrove (*Avicennia marina*) seedlings were also higher in oyster reefs ($p \leq 0.016$ and $p \leq 0.04$, respectively), compared to other habitats, with an average of ≈ 7 ± 1 seedlings ind.m^{-2}.

Table 1. Average ± standard error (SE) and range (min–max) of micro-habitat characteristics obtained from photo-quadrats (n = 96), excavated quadrats (n = 48) and sediment cores (n = 36). This includes measurements associated with the biogenic structures of grey mangroves (*Avicennia marina*), Pacific oyster (*Magallana gigas*) and cockle shells (*Katelysia* spp.). Post-hoc tests denote significant results of Kruskal-Wallis tests and pairwise Dunn's post-hoc comparisons between habitat types ($p \leq 0.05$). Abbreviations: S = bare sediment, M = mangrove forests and O = oyster reefs.

Habitat characteristics	Bare sediment		Mangroves		Oyster reef		Post-hoc tests
	$\bar{x}$ ± SE	Range	$\bar{x}$ ± SE	Range	$\bar{x}$ ± SE	Range	
Habitat cover & mangrove structure densities from photo-quadrats (N = 96)							
Oyster shell %	0.07 ± 0.07	0–2.38	0.63 ± 0.28	0–6.84	31.21 ± 2.87	9.97–76.93	S < M < O
Bare sediment %	99.43 ± 0.20	93.96–100	88.47 ± 1.58	58.43–97.97	60.82 ± 2.84	17.45–81.55	S > M > O
Cockle shell %	0.48 ± 0.19	0–6.04	2.81 ± 1.25	0–38.15	1.51 ± 0.36	0–8.06	S < (M = O)
Mangrove roots %	-	-	7.39 ± 1.08	1.76–25.66	5.59 ± 0.66	1.18–15.51	S < (M = O)
Seedling %	0.02 ± 0.02	0–0.56	0.69 ± 0.24	0–5.11	0.87 ± 0.20	0–4.01	S < M < O
Pneumatophore roots.m^{-2}	-	-	250 ± 28	28–644	258 ± 27	60–624	S < (M = O)
Seedlings ind.m^{-2}	0.1 ± 0.1	0–4	3.1 ± 0.7	0–12	6.6 ± 1.1	0–24	S < M < O
Shell densities & weight from excavated quadrats (N = 48)							
Dead oyster ind.m^{-2}	-	-	0.50 ± 0.50	0–8	169 ± 27	36–468	(S = M) < O
Alive oyster spat ind.m^{-2}	-	-	0.50 ± 0.50	0–8	190 ± 60	0–812	(S = M) < O
Alive adult oyster ind.m^{-2}	-	-	2.25 ± 1.09	0–12	254 ± 32	88–532	(S = M) < O
Cockle shells g.m^{-2}	173 ± 66	0–948	392 ± 241	1–3880	594 ± 103	56–1,704	(S = M) < O
Oyster material g.m^{-2}	-	-	73 ± 40	0–536	13146 ± 1517	5,228–24,176	(S = M) < O
Sediment composition (N = 36)							
soil organic matter %	0.97 ± 0.12	0 .53–1.75	2.17 ± 0.69	0.34–8.75	1.47 ± 0.28	0.53–1.75	S = M = O

The percentage of sediment organic matter was similar across habitats (Kruskal-Wallis: $\chi^2 = 3.297$, $p = 0.192$), comprising 1.53 ± 0.26 percent on average, overall (Table 1). Measured water temperature and salinity across habitats ranged between 16.9 and 23.2 °C and 35 and 44 ppt, respectively.

Between the mangrove and oyster reef habitat treatments, no differences were detected in pneumatophore diameters ($W = 4804$, $p = 0.359$) or aboveground heights ($W = 4074$, $p = 0.306$), which had average (± SE) measurements across sites of 9.9 ± 0.2 mm and 89.4 ± 2.9 mm, respectively (Suppl. material 1, figure S2). We also measured 335 adult and 237 juvenile oysters, which indicated that most individuals were either spat (≤ 5 mm) or adults between 25 and 85 mm, to maximum length of 121 mm (Suppl. material 1: fig. S3A). Of the 2,469 oyster shells counted, 27% were dead 'cups' and 'boxes', with the remaining 73% split between alive adults (42%) and juveniles (31%, Suppl. material 1: fig. S3B).

Excavated quadrat assemblages

We collected 11,500 macrofaunal organisms (excluding oysters) belonging to 64 species and 12 taxonomic classes (Suppl. material 1: table S1). All species were invertebrates, except oyster blennies (*Omobranchus anolius*) (hereafter collectively termed 'invertebrates'). Oyster reefs were associated with 92% of invertebrate species, of which 21 species were exclusively found in this habitat, as compared to mangroves (3 species) or bare sediment (2 species). Most invertebrate abundance was comprised of minute air-breathing snails *Salinator fragilis* and *Hydrococcus brazieri* (33%) and clams *Lasaea australis* and S*cintillua solida* (18%). Ragworms (*Polychaeta* spp.), *S. fragilis* and *S. solida* were the most ubiquitous species, found in > 88% of quadrats. In contrast, the intertidal snails *Austrocochlea constricta* and

Bembicium auratum contributed the greatest combined biomass, representing 31% of total macrofauna weight. Quadrat surveys also detected 28 individuals invertebrates that are cryptogenic and/or non-native species, which were European shore crabs (*Carcinus maenas*), mud creepers (*Batrillaria australis*), Hercules club whelks (*Pyrazus ebenius*) and Sydney cockles (*Anadara trapezia*), 97% of these organisms being surveyed from the oyster reef habitats (Suppl. material 1: table S1).

Excavated quadrats from the oyster reefs typically supported at least two times greater species richness (average ± SE: 23.9 ± 1.2 species), 1.8 times greater densities (1,534 ± 191 individuals.m^{-2}) and 7.6 times more invertebrates biomass (426 ± 84 grams.m^{-2}) than the other two habitats (Fig. 3). ANOVA tests indicated significant differences in the invertebrate richness, density and biomass amongst habitats ($p \leq 0.001$; Suppl. material 1: table S2A); however, post-hoc Tukey's tests indicate these were not significant for invertebrate densities ($p > 0.215$, all pairings; Fig. 3). Both richness ($R^2 = 0.83$) and biomass ($R^2 = 0.83$) were strongly explained by the predictors from the Generalised Linear Models, particularly habitat type, with both metrics also decreasing with increasing distance from the estuary mouth (Suppl. material 1: table S2A). PERMANOVA tests provided strong evidence of habitat differences in the macrofauna community structure ($F = 20.457$, $p = 0.001$), with differences between all habitat combinations ($p = 0.001$, Suppl. material 1: table S3). This is supported by Fig. 4, with distinct clustering of sites by habitat type within the dbRDA plot. These habitat differences in assemblages were strongly correlated to oyster shell (*Magallana gigas*) biomass and pneumatophore densities (Fig. 4). Species most strongly associated with each habitat included isopods (Sphaeromatidae spp.) and little shore crabs (*Brachynotus spinosus*, oyster reefs), Long-legged fly larvae (Dolichopodidae spp., mangroves) and Brazier's hydrococcus (*Hydrococcus brazieri*, bare sediment) based on Dufrene-Legendre analysis (Table 2, Fig. 4).

Remote underwater video nekton assemblages

Remote underwater video (RUV) surveys detected 32 nekton species divided amongst 24 fishes, six decapods and one cephalopod, with a combined abundance (total MaxN) of 3,641 organisms (Fig. 5, Suppl. material 1: table S4A). Nekton were numerically dominated by small pelagic fishes (66.1%), smallmouth hardyhead (*Atherinosoma microstoma*) and blue sprat (*Spratelloides robustus*), followed by King George whiting *Sillaginodes punctatus* (8.3%). Invertebrates comprised 1.4% of abundances and included hermit crabs (*Diogenes senex*), blue swimmer crabs (*Portunus armatus*) and European shore crabs. We observed 29 species from

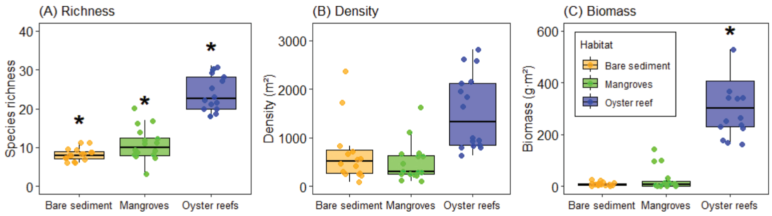

Figure 3. Boxplots showing invertebrate **A** richness **B** density m^{-2} and **C** biomass grams.m^{-2} from excavated quadrat surveys across intertidal habitats (bare sediment, mangrove forests and oyster reefs; n = 48). The boxes represent the 50th percentile (interquartile range), with the median value indicated by a horizontal line within each box. The whiskers extend to the 95th percentile. Overlaid points represent samples. Significant differences between habitats identified from Tukey pairwise comparisons are denoted by asterisk "*".

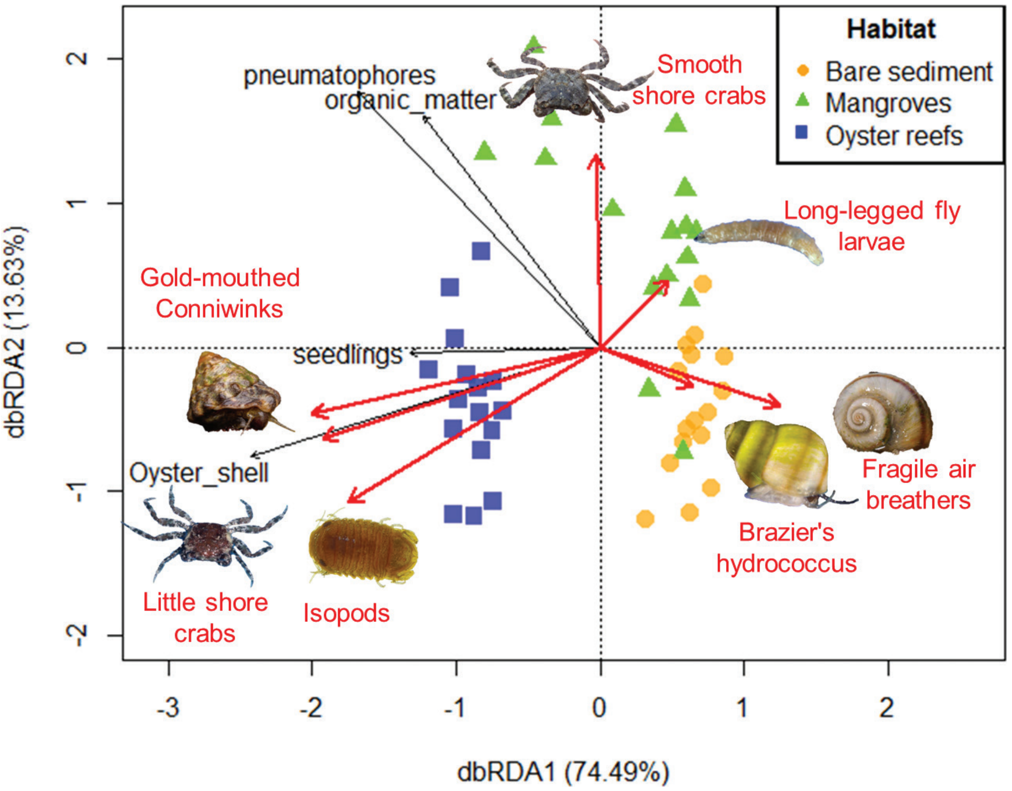

Figure 4. Distance-based redundancy analysis (dbRDA) plots showing the correlation relationships between invertebrate assemblages and environmental predictors associated with the excavated quadrat surveys. Points represent surveys, categorised by habitat. Vectors indicate the direction and strength of significantly correlated ($p < 0.05$) environmental variables (black arrows); and the top indicator species from Dufrene-Legendre indicator analysis (red arrows).

Table 2. Indicator species analysis outcomes, showing the top (≤ 3) species with significant indicator values (IndVal) and their associated *p*-values for different habitat types. *No indicator species were identified from fyke-net samples.

Species	Habitat	IndVal	*p*-value
Excavated quadrat surveys			
Isopods Sphaeromatidae spp.	Oyster reef	0.97	0.001
Little shore crabs *Brachynotus spinosus*	Oyster reef	0.97	0.001
Gold-mouthed conniwinks *Bembicium auratum*	Oyster reef	0.93	0.001
Long-legged fly larvae Dolichopodidae spp.	Mangroves	0.49	0.018
Burrowing shore crab *Leptograpsodes octodentatus*	Mangroves	0.25	0.022
Brazier's hydrococcus *Hydrococcus brazieri*	Bare sediment	0.67	0.023
Fragile air breather *Salinator fragilis*	Bare sediment	0.62	0.001
Remote underwater video system surveys			
Oyster blenny *Omobranchus anolius*	Oyster reef	0.66	0.001
Western striped grunter *Pelates octolineatus*	Oyster reef	0.55	0.007
Black bream *Acanthopagrus butcheri*	Oyster reef	0.48	0.046
Yellowfin whiting *Sillago schomburgkii*	Bare sediment	0.59	0.001
Yelloweye mullet *Aldrichetta forsteri*	Bare sediment	0.48	0.039
Smooth toadfish *Tetractenos glaber*	Bare sediment	0.45	0.008

oyster reefs, including 11 that were unique to this habitat, as compared to bare sediment (2 species) and mangroves (1 species). Southern longfin gobies (*Favonigobius lateralis*), black bream (*Acanthopagrus butcheri*) and King George Whiting were the most ubiquitous species observed, occurring in > 75% of video surveys.

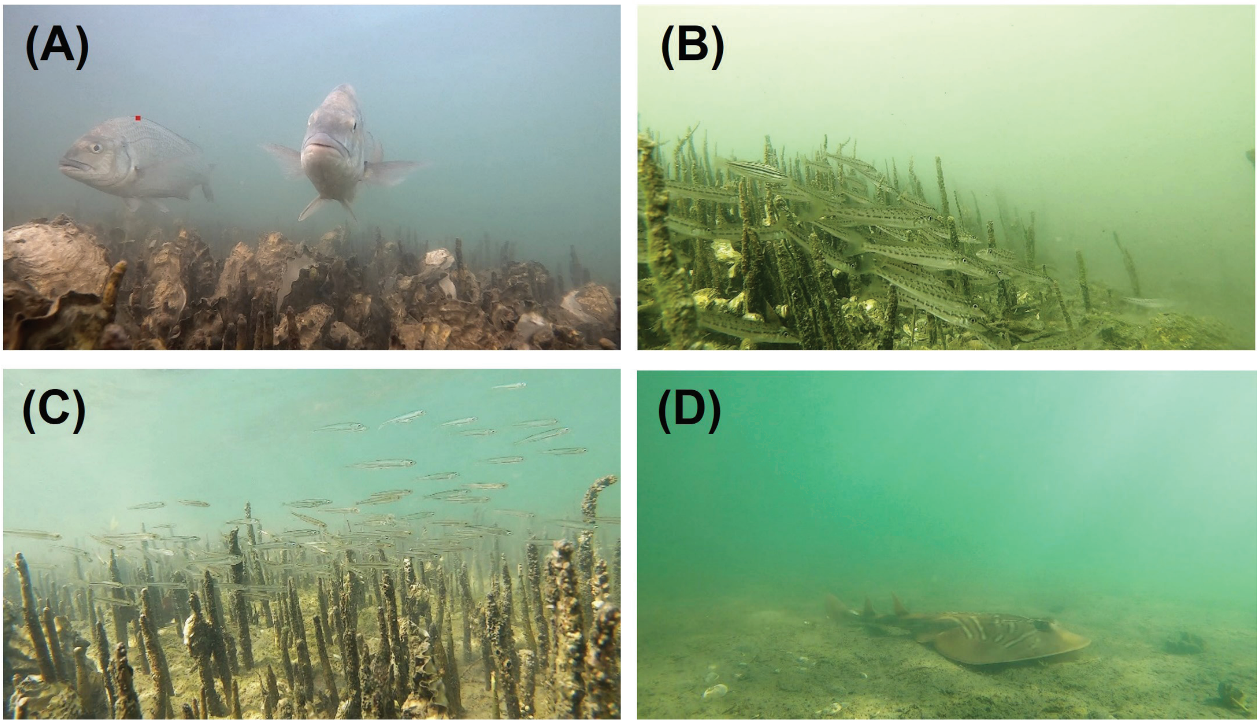

Figure 5. Examples of nekton observed from the remote underwater video (RUV) surveys, including **A** black bream *Acanthopagrus butcheri* **B** juvenile King George whiting *Sillaginodes punctatus* **C** smallmouth hardyheads *Atherinosoma microstoma* and **D** southern fiddler ray *Trygonorrhina dumerilii*.

We observed cryptogenic and/or non-native European shore crabs (n = 4), crested oystergobies (*Cryptocentroides gobioides*, n = 10) and largemouth gobies (*Redigobius microstoma*, n = 1) with 80% of their total abundance derived from oyster-reef surveys (Suppl. material 1: table S4A).

Oyster reefs, on average, were associated with comparatively higher nekton species richness (10.6 ± 0.9 species) and abundances (148 ± 40.2 total MaxN), compared to the other habitats (Fig. 6A, B). Generalised Linear modelling and subsequent Post-hoc Tukey's tests provided strong evidence of greater nekton richness from oyster reefs ($p \leq 0.033$, both) and similar, but lower nekton richness amongst mangrove and bare sediment habitats ($p = 0.893$; Suppl. material 1: table S2B). Additionally, the Generalised Linear Models suggested that total abundances decreased over time during the study period, but increased with increasing distance from the estuary mouth (Suppl. material 1: table S2B). PERMANOVA results indicated habitat differences in the multivariate nekton assemblage from the RUV surveys ($F = 18.39$, $p = 0.001$; Suppl. material 1: table S3), contrasting assemblages from oyster reefs and mangroves ($p = 0.390$), from bare sediment habitats ($p < 0.021$, both). These trends were predominantly driven by indicator species including oyster blennies and western stripped grunters (*Pelates octolineatus*) in oyster reefs and yellowfin whiting (*Sillago schomburgkii*) and yelloweye mullet (*Aldrichetta forsteri*) in bare sediment habitats (Table 2, Fig. 7A). The indicator test did not identify any nekton species that were strongly associated with the mangrove-dominated habitat (Table 2), likely driven by shared occurrence patterns across two or more habitats (Suppl. material 1: table S4A). Assemblage patterns were strongly correlated to trends in the oyster shell biomass, *A. marina* pneumatophore and seedling cover and distance from the estuary mouth (Fig. 7A).

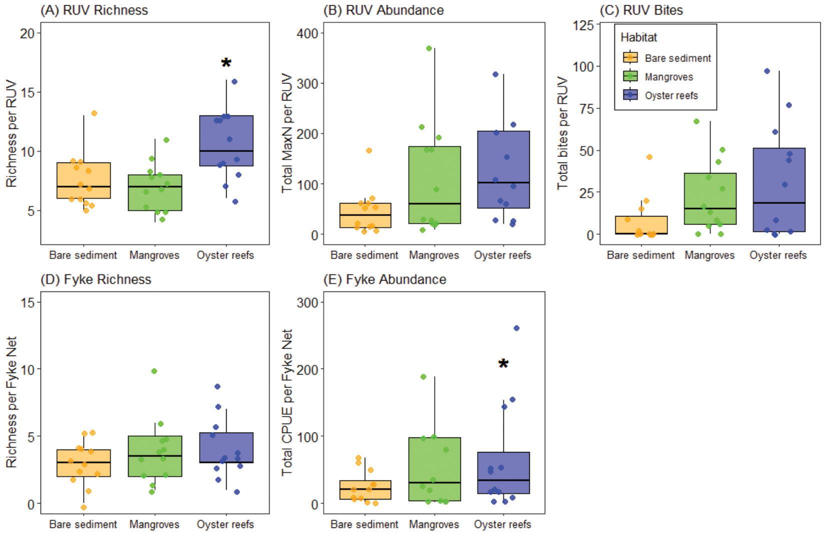

Figure 6. Boxplots showing nekton **A** richness **B** total abundance and **C** bite counts from the remote underwater video (RUV) surveys and **D** richness and **E** total abundance from fyke-net surveys. The boxes represent the 50th percentile (interquartile range), with the median value indicated by a horizontal line within each box. The whiskers extend to the 95th percentile. Overlaid points represent samples. Significant differences between habitats identified from Tukey pairwise comparisons are denoted by asterisk "*".

Fish foraging

Fish foraging behaviour was quantified from 730 bites across 10 different species over the study duration. The most frequent foragers were southern longfin gobies and King George whiting representing 37% and 22% of total observed bites, respectively (Suppl. material 1: table S4A). Most bites were observed in oyster-reef habitats (51%), which, on average, recorded greater bites (30.7 ± 9.9 bites), compared to the mangrove (22.5 ± 6.3) or bare sediment (7.7 ± 4.0) habitats (Fig. 6C). Generalised Linear Models indicated that total bite counts ($R^2 = 0.52$) were best predicted by habitat type and generally increased with increasing oyster shell and pneumatophore cover, as well as closer proximity to the estuary mouth (Suppl. material 1: table S2B). However, the bite counts were variable across samples, with post-hoc Tukey tests not supporting significant pairwise differences amongst habitats ($p > 0.808$, all pairwise tests; Fig. 6C). Feeding behaviour was exhibited by five fish species at the mangrove and bare sediment habitats each and nine species within the oyster reef habitats.

Fyke-net nekton assemblages

Fyke nets caught 2,195 nektonic organisms representing 19 species divided amongst 14 fish, four decapods and one cephalopod (Suppl. material 1: table S4B). The majority of caught nekton were smallmouth hardyheads (49%), southern longfin gobies (38%) and glassgobies (*Gobiopterus semivestitus*, 5%). Two cryptogenic species, crested oystergobies (n = 2) and exquisite sandgobies (*Favonigobius exquisitus*, n = 1), were also collected, exclusively from oyster reef habitats. Of the caught fish species, 58% were found across multiple habitat types, with the total species richness varying across bare sediment (9 spp.), mangrove (13 spp.) and oyster reefs (15 spp.) habitats.

Generalised Linear modelling found that nekton richness ($R^2 = 0.51$) obtained from fyke nets increased with closer proximity to the estuary mouth and increasing

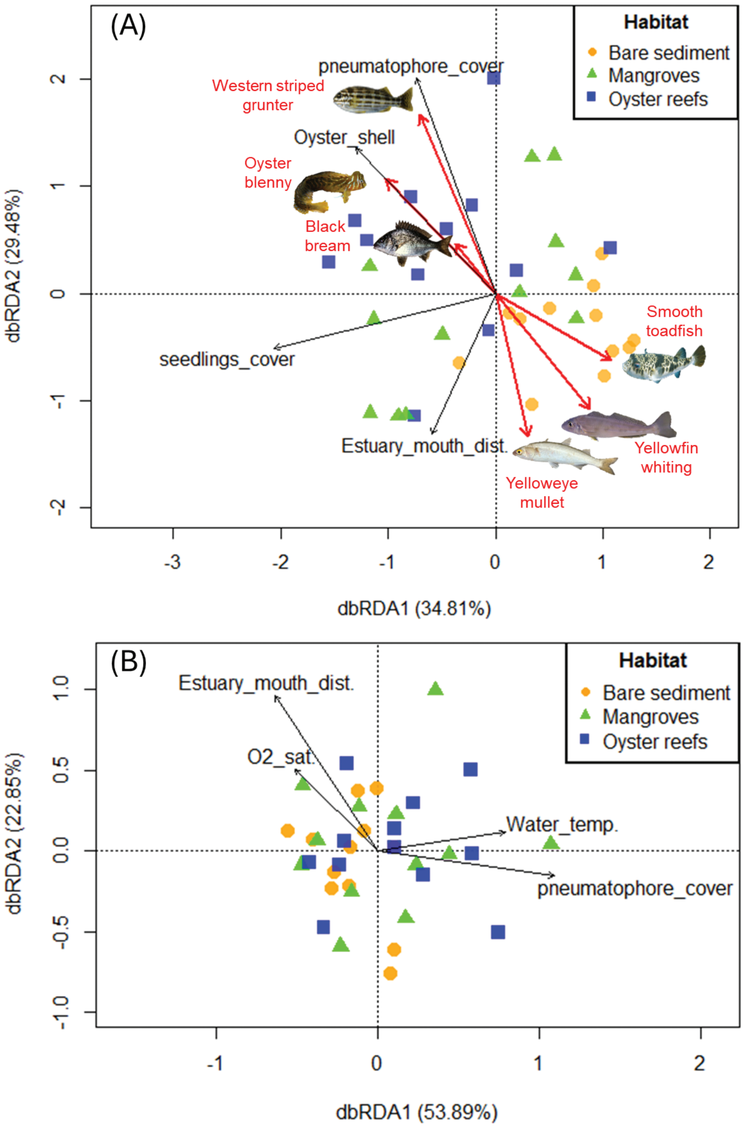

Figure 7. Distance-based redundancy analysis (dbRDA) plots showing the correlation relationships between environmental predictors and the nekton assemblages surveyed using **A** remote underwater video systems (RUV) and **B** fyke nets. Points represent surveys, categorised by habitat. Vectors indicate the direction and strength of significantly correlated ($p < 0.05$) environmental variables (black arrows); and the top indicator species from Dufrene-Legendre indicator analysis (red arrows).

A. marina seedling cover, with no habitat differences detected (Fig. 6D, Suppl. material 1: table S2C). In comparison, total abundances ($R^2 = 0.55$) was best predicted by habitat type and generally increased with increasing oyster (*Magallana gigas*) shell, with the opposite pattern for *A. marina* seedling cover (Suppl. material 1: table S2C). Post-hoc tests contrasted oyster reef assemblages from the other habitats ($p < 0.002$; Fig. 6E), with average (± SE) catches varying across mangroves (92.9 ± 45.5 CPUE), oyster reefs (65.0 ± 23.1 CPUE) and bare sediment (25 ± 6.7 CPUE) habitats. PERMANOVA of the multivariate nekton assemblages from the fyke nets did not support habitat differences (PERMANOVA: $F = 0.720$, $p = 0.775$; Suppl. material 1: table S3), which is reflected by the lack of clustered points in the dbRDA plot (Fig. 7B). While estuary mouth distance and pneumatophore densities were important predictors for the multivariate assemblages (Fig. 7B), we were unable to identify significant indicator species, due to either insufficient sample size or species occurrence across multiple habitats (Table 2).

Nekton length measurements

Length measurements were obtained from 386 individual nekton, from 19 species caught using fyke nets (Table 3). Adult nekton mainly comprised Atherinidae and Gobiidae species, while juveniles included fisheries-targeted King George Whiting (range 72—120 mm total length) and blue swimmer crabs (range 4—25 mm carapace length). Significant habitat differences in size distributions were only identified for southern longfin gobies ($\chi^2 = 7.34$, $p < 0.03$), smallmouth hardyheads ($\chi^2 = 13.24$, $p < 0.002$) and glassgobies ($\chi^2 = 9.25$, $p < 0.01$; Fig. 8). The oyster reefs were associated with larger individuals of each species (average ± SE: 30.8 ± 0.9 mm,

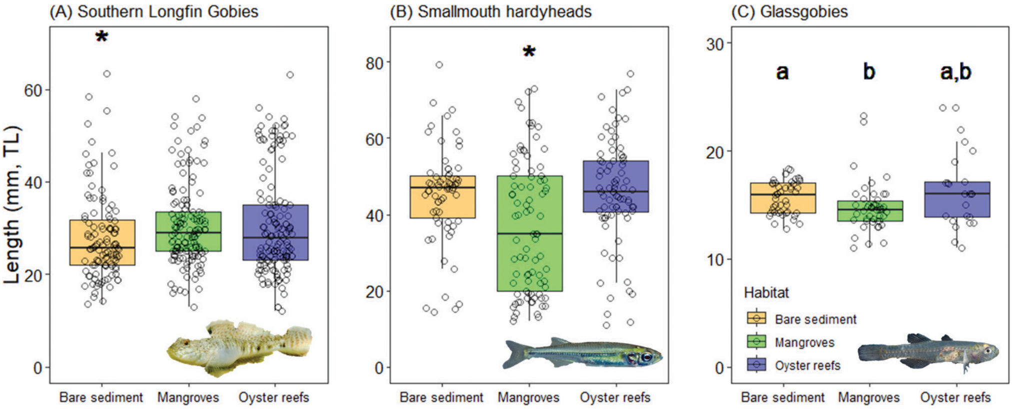

Figure 8. Boxplots of length frequency distribution of total length (mm) for **A** Southern longfin gobies, *Favonigobius lateralis* **B** Smallmouth hardyheads, *Atherinosoma microstoma* and **C** Glassgobies, *Gobiopterus semivestitus*. The boxes represent the 50th percentile (interquartile range), with the median value indicated by a horizontal line within each box. The whiskers extend to the 95th percentile. Overlaid points represent samples. "*" and "a, b" denote results of Dunn's post hoc tests comparing length distributions by habitat type.

Table 3. Total number of individuals per species measured from each habitat (N), body length (millimetres), type (total length (TL), carapace length (CL) or mantle length (ML)) and range (min-max) for each species caught in the fyke nets from Torrens Island.

		Habitat type					
		Bare sediment		**Mangroves**		**Oyster reefs**	
Species name	**Length (mm)**	**Total n**	**Length Range**	**Total n**	**Length Range**	**Total n**	**Length Range**
Acanthopagrus butcheri	TL					1	12.2
Aldrichetta forsteri	TL	6	85.0–120.6				
Atherinosoma microstoma	TL	65	14.4–79.4	85	12.0–73.0	78	11.0–77.0
Bathygobius krefftii	TL			2	42.0–54.0	6	47.2–58.0
Cryptocentroides gobioides	TL					2	32.0–44.0
Diogenes senex	CL	1	4.9–9.4	6	3.6–9.4	9	5.0–10.0
Favonigobius lateralis	TL	107	13.6–63.2	134	13.0–57.9	145	12.0–63.1
Favonigobius exquisitus	TL					1	57
Gobiopterus semivestitus	TL	44	12.8–18.4	46	11.0–23.3	25	11.0–24.0
Kestratherina esox	TL	7	48.0–81.0	5	65.7–84.9	21	43.4–84.8
Neoodax balteatus	TL			1	67.0		
Palaemon intermedius	CL			1	3.0	1	7.0
Pelates octolineatus	TL					1	119.5
Penaeus latisulcatus	CL	1	8.0				
Portunus armatus	CL			10	4.0–19.0	27	6.0–25.5
Pseudaphritis urvillii	TL			1	121.0		
Pseudogobius olorum	TL			5	38.0–55.0	3	24.0–43.0
Sillaginodes punctatus	TL	13	73.9–117.5	6	85.0–120.0	1	72.0
Xipholeptos notoides	ML	2	5.0	1	11.0	7	4.0–11.0

45.2 ± 1.6 mm, 16.4 ± 0.7 mm TL, respectively). On average (± SE), southern longfin gobies were smallest from the bare sediment habitat (28.1 ± 0.9 mm TL), while smaller individuals of the other two species were associated with the mangrove habitat (36.8 ± 1.9 mm, 14.8 ± 0.34 mm, respectively TL) (Fig. 8). For the remaining species, there was insufficient evidence to indicate habitat differences in length measurements, which could be attributed to low sample size or similar length classes present across multiple habitats (Table 3).

Discussion

Despite the ecological risks of non-native habitat-formers globally (Rilov et al. 2024), such as Pacific oysters *Magallana gigas*, our knowledge on the interaction between non-native oyster reefs and invaded ecological systems is poor (Hansen et al. 2023). In contrast, endemic oysters reefs are increasingly associated with valuable ecological functions including habitat provisioning and fish production (Gilby et al. 2018; Connolly et al. 2024), with further enhancements via facilitation cascades with other habitat-formers (Gagnon et al. 2020; Thomsen et al. 2022). We found that, despite its non-native status, ‘oyster reefs’, formed from the nested co-occurrence of wild Pacific oyster *M. gigas* and grey mangroves *A. marina*, were generally more biodiverse compared to our uninvaded bare sediment or mangrove habitats. These results indicate that non-native oyster reefs may enhance the availability and range of ecological niches and functions, potentially similar to native shellfish reefs.

Pacific oyster habitat characteristics

Within the past decade, non-native Pacific oysters have extensively colonised the Port River-Barker Inlet Estuary. The *M. gigas* population transitioned from sparse individuals (Wiltshire et al. 2010), to dense biogenic oyster reefs, containing an average of 254 alive adults.m^{-2} and 13.2 kg.m^{-2} of oyster material, as identified in our study. These densities exceed those of former endemic oysters (*Ostrea angasi*) which formed reefs at densities of > 50 alive adults.m^{-2} (Gillies et al. 2020). Pacific oyster aggregations modified the habitat characteristics of colonised mangrove forests, establishing complex, biogenic shell matrices. The consolidated surfaces of mangroves can facilitate secondary foundation species, such as epifaunal oysters, by provisioning settlement substrate (Gagnon et al. 2020). Oyster ‘reefs’ within mangrove forests then arise from successive recruitment and shell accumulation, supported by intraspecific and interspecific facilitation interactions (Reeves et al. 2020) and hydrological influences that aggregate oyster recruits.

Despite high Pacific oyster densities potentially affecting mangroves by limiting gas exchange (Cannicci et al. 2008), similar pneumatophore densities and morphologies were observed in our study, regardless of oyster presence. This could be due to oyster spat initially settling on already mature mangrove root systems, with subsequent settlement preferencing the adult shells of established oyster reefs (Bishop et al. 2012), rather than inhibiting growing pneumatophores. Pacific oysters are, therefore, not substrate limited in this system, based on the presence of extensive pneumatophores and disarticulated cockle shell that were free of oysters. The young age of the reefs (< 10 years) means that negative interactions with established mangroves may not occur until an oyster density threshold is surpassed, which may not manifest for decades (Bazterrica et al. 2022). While the ecological impacts of Pacific oysters are likely restricted to the fringing shoreline based on tidal inundation patterns, our study provides a baseline suitable for future assessments of these sites to understand long-term variability in species interactions and habitat composition.

Allochthonous material including disarticulated shell material and mangrove debris were found in greater quantities from the oyster reef habitats. Buoyant material, such as mangrove propagules and leaf litter disperse with wave currents and subsequent tidal action (Yun et al. 2022). By modifying the shoreline topography, Pacific oyster shells may trap and retain material, including mangrove propagules (McClenachan et al. 2021) and provide physical protection to newly-settled mangrove recruits (Gagnon et al. 2020). This could explain the high *A. marina* seedling abundances associated with the Pacific oyster reefs. Over longer timeframes, enhanced mangrove recruitment around the *M. gigas* may facilitate mangrove forest expansion, potentially to the detriment of the oysters, as observed in intertidal American oyster *Crassostrea virginica* reefs (McClenachan et al. 2021). While we also hypothesised that oysters may support mangroves through biodeposition, we did not detect habitat differences in soil organic matter in our study. This could be due to: (1) rapid decomposition of material by high macroinvertebrate densities, (2) resuspension and transport of fine organic material during tidal flushing or (3) confounding influences of microbial and microalgae mats across sites (Kristensen et al. 2008). Further investigations are, therefore, needed to understand how *M. gigas* may influence co-existing vegetation and associated fluxes in organic matter.

Habitat value for fish and invertebrates

Oyster reefs in the Port River-Barker Inlet Estuary were mainly found to benefit nekton species that live on or feed near the benthos. Cryptobenthic oyster blennies and Gobiidae species were reef 'residents' within this habitat, consistent with previous studies that have identified their use of disarticulated bivalve shells as nesting sites and refugia throughout the tidal cycle (Hammer 2014; Martinez-Baena et al. 2022). Furthermore, high abundances of fisheries-targeted black bream and western striped grunters could be attributed to foraging behaviour, preferencing the oyster reefs to feed on associated invertebrates, particularly crustaceans and bivalves (Sarre et al. 2000; Potter et al. 2022). While fyke nets were less sensitive to detecting species-specific distribution trends, the larger small-bodied fishes from the oyster reef habitats could indicate size-based habitat preferencing or favourable environmental conditions. Nekton community composition from the RUV surveys was most similar between sites dominated by mangroves, with and without Pacific oyster aggregations. We hypothesise that this was driven by species-specific trends and some functional redundancy between habitats resulting from the shared co-occurrence of *A. marina* structures like pneumatophores. For example, small pelagic fishes (e.g. Atheriniformes) were common to both habitats and may access fringing mangroves for refugia at high tide (Henkens et al. 2022). Given that we observed foraging behaviour across habitat types, assessments such as stomach contents, predation assays or stable isotope analysis, could be valuable for understanding trophic linkages of these habitats and associated species (Martinez-Baena et al. 2023; Martin et al. 2024), including.

During our study, seven non-native and/or cryptogenic species were sampled exclusively or in greater numbers from the oyster reefs, including the first confirmed specimens of the Hercules club whelk (*Pyrazus ebeninus*) and exquisite sandgoby (*Favonigobius exquisitus*) in South Australia. Thus, Pacific oyster aggregations may facilitate secondary invasions by other non-native species by creating favourable habitat conditions (Reise et al. 2023). For example, the European shore crab (*Carcinus maenas*) and crested oystergoby (*Cryptocentroides gobioides*) from eastern Australia are strongly associated with intertidal shellfish ecosystems in their respective native ranges (Troost 2010; Martinez-Baena et al. 2022). These species, therefore, should respond positively when introduced into similar environmental conditions (O'Loughlin and Green 2017). The presence of non-native species alongside native counterparts at our study site could result in inter-specific competition of shared resources and direct predation (Hammer 2014; Gallardo et al. 2016). Of particular concern are *C. maenas*, which may indirectly facilitate Pacific oysters by consuming their competitors. They exhibit dietary preferences for mussels over oysters (Joyce et al. 2020), posing a threat to the native *Brachidontes* and *Xenostrobus* mussels found at our sites (Campbell et al. 2019).

Our combination of excavated quadrat, RUV and fyke-net surveys in dynamic intertidal habitats provided comprehensive information about estuarine fish and invertebrate habitat distributions across multiple spatial-scales (Stein et al. 2014). Fine-scale variation in benthic structure was found to strongly influence invertebrates and, while we did not detect consistently high total invertebrate densities in oyster reefs (Grabowski et al. 2005), our results suggest that these habitats support enhanced invertebrate species richness and biomass and, therefore, potentially offer diversified foraging resources (Martinez-Baena et al. 2023). These results conform to previous assessments of oyster reef associated invertebrates (Bishop et al. 2012; McAfee et al. 2016), which may benefit from enhanced shelter, settlement surfaces and trophic resources.

Both RUV and fyke nets have been applied in intertidal shellfish ecosystems elsewhere in the world (reviewed in Martin et al. (2024)) and offer complimentary information when used in combination (Orfanidis et al. 2021). Specifically, unbaited RUV surveys target large-bodied species and record natural behaviour, while fyke nets enhance surveys and measurements of small-bodied nekton (e.g. Orfanidis et al. (2021); Martinez-Baena et al. (2022)). Similarly, by sampling both the outgoing tide (i.e. RUV and fyke nets) and at low tide using extractive quadrats, it is possible to infer the tidal movement of different species and identify resident species that are strongly associated with habitats throughout the tidal cycle. This approach is particularly relevant for intertidal ecosystems as transient species vary their habitat use, based on tidal accessibility and resources availability (Grabowski et al. 2005; Martin et al. 2024). For example, nekton metrics in our study were highly variable within habitats and over the study period, suggesting that broader spatio-temporal influences, such as environmental gradients or seascape composition, could be impacting species distributions and behaviour across habitats (e.g. Jones et al. (1996); Perry et al. (2023)). This is further supported by distance from the estuary mouth being an important predictor for fish and invertebrate metrics, which may have confounded habitat patterns across sites. We recommend that practitioners assessing intertidal oyster reefs incorporate multiple sampling methods to effectively monitoring biodiverse assemblages and to consider different environmental variables that may influence or limit different approaches.

In contrast to other non-native species, invasions by bivalves generally facilitate enhanced biodiversity through positive ecological interactions, such as increasing habitat complexity and ameliorating stresses (Guy-Haim et al. 2018). Oyster reefs are known to benefit fish and invertebrate communities by provisioning high value foraging and nursery habitat and reducing abiotic and biotic stressors (e.g. predation, desiccation; Reeves et al. (2020); zu Ermgassen et al. (2021)). Our results concur with recent studies that have reported positive oyster reefs interactions with fish and invertebrate communities in mangrove-dominated systems (e.g. Gilby et al. (2019); Martinez-Baena et al. (2022); Perry et al. (2023)). For example, Perry et al. (2023) found that the fish species richness and harvestable fish abundance in structured habitats positively responded to oyster cover. Similarly, McAfee et al. (2016) showed that oyster aggregations in mangrove forests facilitated elevated invertebrate richness and abundances, compared to mangroves without oysters. These results can be attributed to facilitation cascades and other positive interactions resulting from the combination of mangroves and oysters, which synergistically enhance the diversity and availability of niches and resources (Stein et al. 2014; Thomsen et al. 2022).

Management implications of non-native oyster reefs

Our findings indicated that non-native oyster reefs are influencing species and assemblage structure, but generally had beneficial impacts such as enhanced biodiversity and positive associations with fisheries-targeted species. While not quantified, these reefs may support other valuable socioecological outcomes including nutrient sequestration, enhanced water visibility and human shellfish harvesting (McAfee and Connell 2021; Hansen et al. 2023). Subsequently, in regions were *M. gigas* are naturalised, such as northern Europe, the oysters have supported the development of new ‘gastro-tourism’ opportunities (Mortensen et al. 2019) and have been adopted in shoreline stabilisation structures (Walles et al. 2016). This has been partially supported by the functional similarities of *M. gigas* to diminished native bivalves and subsequent recovery of lost ecological functions (Zwerschke et al. 2019; McAfee and Connell 2021). As highlighted by Howie and Bishop (2021), non-native oysters may be considered for the restoration of ecological services, particularly in naturalised regions where analogous native species have failed to recover or adapt to modified environmental conditions. However, *M. gigas* may only partially restore functions of lost subtidal shellfish reefs (e.g. *O. angasi* in South Australia) and potentially establish novel, yet beneficial conditions in regions that historically lacked comparable habitats (Guy-Haim et al. 2018; McAfee and Connell 2021). In an anthropogenically changing world, the positive benefits of non-native habitat formers must increasingly be studied and considered to conserve and enhance ecological services (Rilov et al. 2024).

The capacity of non-native habitat-formers to modify ecological services and community composition poses a management challenge for scientists and policy-makers (Rilov et al. 2024). Beyond the risk of secondary invasions, Pacific oysters can induce other socioecological impacts including changes in landscape aesthetics, channel navigation and aquaculture interactions (e.g. disease transmission, genetic exchange; Herbert et al. (2016); Hansen et al. (2023)). Furthermore, positive ecological benefits to endemic species may change through time with increasing *M. gigas* dominance or induce unexpected negative cascades in adjacent habitats. Efforts to mitigate Pacific oysters is further complicated by its dual status as both an aquaculture species and marine pest (Martínez-García et al. 2022). Cultivation of *M. gigas* outside their native range can support non-native reef establishment through repeated seeding and genetic enhancement from aquaculture stock (McAfee and Connell 2021). Furthermore, efforts to completely eradicate and prohibit *M. gigas* aquaculture are unlikely, due to the negative socio-economic impacts and low likelihood of success (Troost 2010; Hansen et al. 2023). Subsequently, management of non-native oyster reefs needs to be evidence-based with consideration of both favourable and undesirable socioecological outcomes within the local regional context (Martínez-García et al. 2022). For example, *M. gigas* expansion into vulnerable and protected ecosystems could be mitigated by regulating aquaculture permissions and implementing harvesting regimes (Hansen et al. 2023). In anthropogenically modified estuaries, however, we expect that non-native oyster reefs may provide more socioecological benefits compared to potential harm, but these habitats require monitoring, regardless of the mitigation strategy.

Conclusion

Non-native habitat-formers, such as Pacific oysters *Magallana gigas*, can pose a significant socioecological challenge for managing coastal ecosystems. In this study, we evaluated the ecological interactions of non-native *M. gigas* on mangrove forest biodiversity, particularly effects on fish communities. Our findings indicate that, despite their non-native status, *M. gigas* can facilitate positive interactions with grey mangroves, leading to subsequent enhancements in fish and invertebrate diversity, prey availability and foraging opportunities. We also identified that oyster reefs supported fisheries-targeted species and may facilitate habitat conditions favourable to a greater range of species and functions. However, oyster reefs facilitated both native and non-native species, including previously undocumented species. Thus, we describe potential ecological outcomes of non-native habitat-formers and outcomes from the global expansion of non-native Pacific oyster reefs in vegetated coastal estuaries.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

This study was conducted following the ethical standards of Flinders University and relevant national and international laws. Necessary permits were obtained, including animal ethics approvals from Flinders University (5635 & 5642), a scientific research permit from the SA Department of Environment and Water (DEW) (M27219-1), and a Ministerial permit from The Department of Primary Industries and Regions, South Australia (PIRSA) (ME9903230).

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Author contributions

Bradley Martin, Ryan Baring, Charlie Huveneers and Simon Reeves conceptualised the idea and methods for this study. Bradley Martin led the fieldwork, sample processing and initial data analysis. Bradley Martin led the writing and created the figures and Ryan Baring, Charlie Huveneers and Simon Reeves provided crucial contributions to the manuscript. Ryan Baring, Charlie Huveneers and Simon Reeves supervised the project. All authors give their approval for the publication of this manuscript in its final support.

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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 figures and tables

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Data type: docx

Explanation note: **figure S1.** Collinearity matrices for environmental predictors related to ecological patterns. **figure S2.** Boxplot of length frequency distribution of grey mangrove (*Avicennia marina*). **figure S3.** Frequency histogram. **table S1.** List of macrofauna species obtained from the extractive quadrats. **table S2.** Results of generalised linear models. **table S3. A** PERMANOVA and post-hoc pairwise outputs. **table S4.** List of nekton species identified.

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Research Article

Are range limits concordant with climatic niche requirements in alien plants? Leguminous invasive plants as case study, along a latitudinal gradient, central Chile

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Abstract

How do species reach the limits of their distribution and what prevents their continued expansion beyond these ranges? Exotic plant species represent a natural experiment to answer these questions. If climate is the limiting factor, then one would expect a matching between the observed range limit for a species and the range limit predicted by its climatic niche. If there is no matching, then other factors such as dispersal limitation, competition or facilitation come into play. In this work, the predicted and observed range limits for eight exotic legume species were compared: *Acacia dealbata*, *Acacia melanoxylon*, *Cytisus striatus*, *Teline monspessulana*, *Ulex europaeus*, *Lotus corniculatus*, *Trifolium suffocatum* and *Vicia villosa*, in a latitudinal gradient in Chile. For the estimation of the observed range limit (North and South), absence/presence data were obtained from 30° to 43.1° south latitude. For the estimation of the predicted range limits, GBIF presence data were used to construct the global climatic niches, identifying suitable climatic zones (presences) and unsuitable climatic zones (absences). With this information, presence probability models were constructed with hierarchical Huisman-Olff-Fresh (HOF) regression, from which the predicted range limits (North and South) were obtained. Our results suggest that the species *Acacia dealbata* and *Cytisus striatus* have reached their predicted edge at the northern and at the southern end of the gradient. The rest of the species have not yet reached this limit across both geographic edges. At the southern end of the gradient, most species have not reached the limit predicted by the climatic niche; except for *Cytisus striatus* whose observed range limit is higher than predicted. Factors other than climate are discussed to explain the discrepancies between observed and predicted range limits.

Key words: Biogeography, climate, legumes, niche requirements, range limits

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Introduction

Charles Darwin (1957) provided the first hypothesis to understand the factors that set limits to species geographic distribution; he proposed that abiotic factors (climate) are important at the abiotic stressful limits of distribution while species biotic interactions become more important at the more benign extreme of the distribution. Nowadays, this framework plays a central role in modern biogeography (Gaston 2003; Sexton et al. 2009) and is assumed a ubiquitous biogeographic pattern (Louthan et al. 2015). New reformulations have proposed to understand range limits of species (Ettinger and HilleRisLambers 2017; Sirén and Morelli 2020; Paquette and Hargreaves 2021), however, they maintain the basic concepts proposed by Darwin.

Hargreaves et al. (2014) proposed a hierarchical approach to understand range limits using the climatic niche of species. This framework predicts the climatic niche limits (CNL) i.e. the geographic limits predicted from climatic niche, and compares it with the range limit (RL), i.e. the observed limits in the extremes of distribution. If RL - CNL = 0, populations located at the limit range, perfectly match between climatic niche requirements and the extreme of distribution; if RL - CNL < 0, beyond RL there are suitable habitats, but they cannot be colonized by species due to dispersal limitation, introduction time or negative biotic interactions (competition, predation); if RL – CNL > 0, in RL populations are occupying unsuitable habitats, they are sink populations which must be continuously supplemented by individuals from source populations in order to persist. Another explanation is that species establish positive interactions with other species, allowing expansion due to facilitation with other plant (Badano et al. 2007; Arredondo-Núñez et al. 2009). Biological invasions provide useful insights to learn about dynamics of species range limits (Sexton et al. 2009). Given that invasive species may have colonized habitats quite different to those existing in their native ranges, we have a natural experiment to examine whether the new range limits depart from expectations from climatic requirements (Keane and Crawley 2002; Gaston 2003; Goncalves et al. 2022).

Climatic niche analysis has been prolifically used to understand the biogeography of biological invasions (Peterson 2003; Thuiller et al. 2005; Broennimann et al. 2007; Reed et al. 2008; Gallagher et al. 2010; Peña-Gómez et al. 2014; Cabra-Rivas et al. 2016). The global climatic niche enables exploration of the climatic potentialities of species using the totality of occurrences recorded for the species across invaded regions (Gallien et al. 2012; Taucare-Ríos et al. 2016). In this study, we assessed if RL is within the variation of CNL using global climatic niche. These methodologies were applied to a set of eight exotic leguminous plants species, invasive in central Chile, and in other regions of the world (Quiroz et al. 2009). Finally, we also discussed its applicability to other biogeographic situations, and to what extent the hypothesis proposed by Hargreaves et al. (2014) give us clues about the geographic dynamics of these eight species.

Methods

The setting

Central Chile concentrates an interesting vascular flora; due to its high degree of endemism and the intense deterioration of ecosystems, this region has been considered a “hotspot” of biodiversity (Armesto et al. 1998; Myers et al. 2000). The notable climatic latitudinal gradient existing in central Chile i.e, decrease of temperatures and an increase of precipitations with latitude has been largely documented in diverse studies (Di Castri and Hajek 1976; Di Castri 1991; Garreaud et al. 2009; Carretier et al. 2018), hence it constitutes an ideal scenario to conduct a natural experiment to test biogeographic hypothesis. We will use this climatic gradient to examine distribution responses of invasive species belonging to the Family Leguminosae along central Chile.

Chile has approximately 690 species of introduced plants (15% of the total flora), being 70% of them of Eurasian origin (Arroyo et al. 2000). These species are spreading into areas with native vegetation, thus affecting the composition and structure of natural communities (Pauchard and Alaback 2004; Bustamante and Simonetti 2005). Approx. 60% of human population is concentrated in central Chile. There is an intensive land use, deforestation and habitat fragmentation, all factors that are regarded as the drivers of biological invasions (Arroyo et al. 2000). During the last years, studies of plant invasion in central Chile have increased significantly (Arroyo et al. 2000; Sax 2002; Pauchard and Alaback 2004; Bustamante and Simonetti 2005; Castro et al. 2005; Peña-Gómez and Bustamante 2012; Fuentes et al. 2014; Peña-Gómez et al. 2014; Montecino et al. 2016). To date, we have a reasonable knowledge about the diversity of exotic species in Chile, however, their biogeography is quite limited (Fuentes et al. 2013, but see Peña-Gómez et al. 2014; Montecino et al. 2016). Our database about the presence of invasive plants for Chile is limited, and the estimation of range limits is only qualitative and at a very coarse spatial scale. Fuentes et al (2013) presented an update about the magnitude of plant invasion in Chile. This information, concomitantly with a local book (Fuentes et al. 2014) was used to select the eight exotic species of this study which are described ranging between 30° and 42° latitude.

Among the numerous exotic species recorded for central Chile (Fuentes et al. 2014), it was decided to work with exotic trees, shrubs, and herbs of the Family Fabaceae (Leguminoseae). These species are regarded as invasive in different parts of the world (Ndlovu et al. 2013; Richardson et al. 2015) and most of them have produced significant ecological impacts in Chile (García et al. 2014, 2015). Taxonomically, these species are well known, their distribution has been documented for central Chile and are conspicuous components of anthropogenic landscapes. The species selected for this study are *Acacia dealbata*, *Acacia melanoxylon*, *Cytisus striatus*, *Teline monspessulana*, *Ulex europaeus*, and *Lotus corniculatus* as well as two herbaceous species, *Trifolium suffocatum* and *Vicia villosa*.

Global climatic niche

Global occurrences data (presences) for the eight exotic species were downloaded during 2019 using the *occ2df* function from the *spocc* package in R (version 0.7.0), which retrieves geographic data for species from several databases as Global Biodiversity Information Facility (**GBIF**), the Atlas of Living Australia (**ALA**), Biological Information Serving Our Nation (**BISON**), EcoEngine, Integrated Digitized Biocollections (**iDigBio**), and iNaturalist (**iNat**). Occurrences were selected if they had a georeferencing error of less than 1 km. To avoid redundancy, duplicate records across databases were identified and removed during data processing (Suppl. material 1 in which we show global occurrences for species). Local occurrences were recorded from 30° to 43° south latitude (Fig. 1), using two transects, one located along the coast and the other, at the central valley. We disposed plots (2 × 50 m), placed along the verge of secondary or tertiary roads, with low management practices; roads are adequate sampling sites as they are the most obvious corridors for the spread of invasive species (Von der Lippe and Kowarik 2008; Barros and Pickering 2014; Van Der Ree et al. 2015). Each plot was located every 10 km, encompassing a total of 264 plots (132 plots per transect). We collected plant samples for further identification in the lab. From this information,

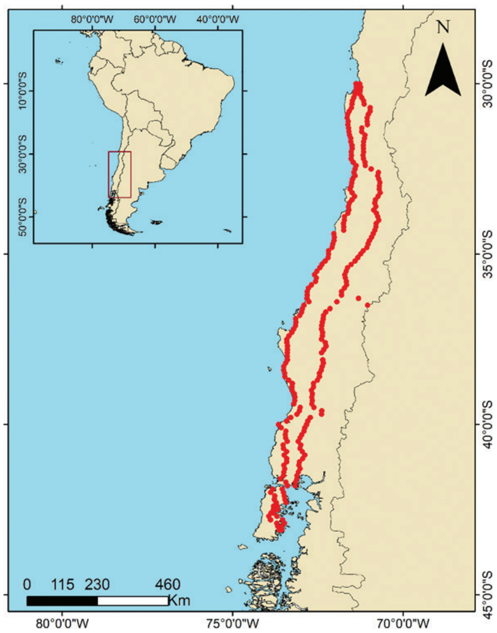

Figure 1. Graphic representation of the points sampled (red) to register the presence/absence of the eight exotic species of this study, across central Chile.

the observed Northern and Southern Range Limits (NRL and SRL respectively) was estimated, each properly georeferenced; they were obtained empirically, recording the last presence for each species at the extremes of latitudinal gradient.

For the estimation of global climatic niche, the climatic grid procedure was used (Broennimann et al. 2012). This method allows the visualization of the climatic niche in a multidimensional space, obtained from Principal Component Analysis (PCA). Specifically, this method was used by means of a data treatment in which: (i) 10000 geo-referenced random points were generated to depict the global climatic environment; (ii) 5300 geo-referenced random points were generated to depict the climatic environment in Chile, through QGIS (version 3.6.1); iii) for climatic characterization and from each of the random points, climatic variables were obtained from Worldclim 2.0, at 1 km2 resolution (Fick and Hijmans 2017), this database includes 19 climatic variables of precipitation and temperature, averaged from 1970 to 2000.The climatic grid procedure (Broennimann et al. 2012) is basically a PCA, which has the advantage of transforming a number of correlated variables into a small number of uncorrelated linear combinations of the original variables (principal components). Unlike other modeling approaches, PCA does not require pre-selecting climatic variables, as the principal components themselves could be used as predictors, reducing the dimensionality of the dataset but maintaining the same information of the original climatic variables (Sillero et al. 2021). Finally, climatic data were correlated with random points through PCA, thus generating the climatic grid (Broennimann et al. 2012). Four regions were identified in the climatic grid (multivariable climatic space): (i) global species occurrences which represent global niche; (ii) the 10.000 random points, depicting the global climatic environment; (iii) local species occurrence which represents local niche in Chile; (iv) the 5300 random points depicting the climatic environments in Chile (Fig. 2). For the purposes of this study, we focus on regions (i) and (iv). Using these two regions, we could define suitable and unsuitable climatic habitats in central Chile; suitable climatic habitats occur in the intersection between

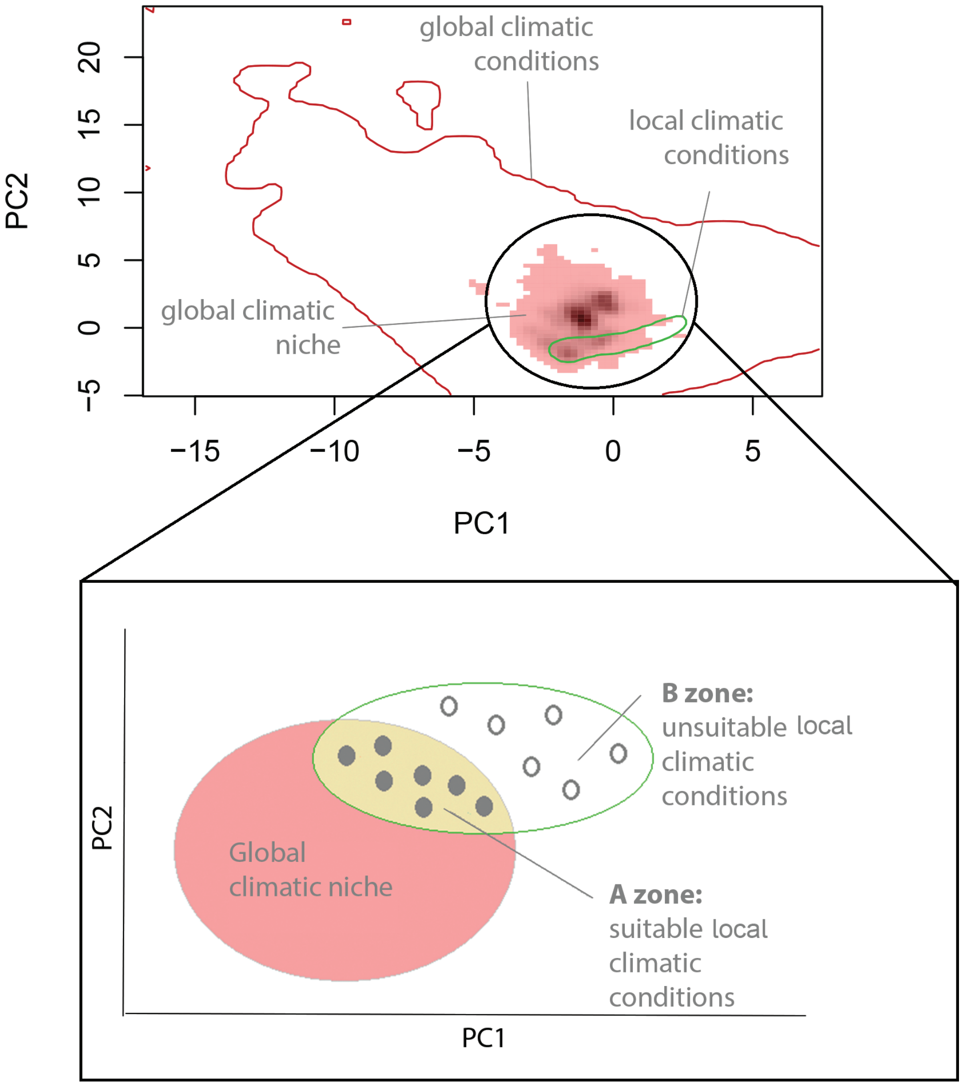

Figure 2. Graphic representation of the climatic grid and global climatic niche to identify suitable and unsuitable habitats **A** PCA with global climatic niches, intersected with the regional scale (study area in central Chile) climate niche. Pink cells: global climatic niche; green line: local climatic conditions in central Chile; red line: global climatic conditions **B** identification of suitable (black points) and unsuitable conditions (white points) for the species in the study area in Chile. Figure obtained from Goncalves et al. (2022) with permission of the authors.

the global climatic niche and the climatic environment in Chile while unsuitable climatic habitats occur in the climatic environment of Chile that is outside the global climatic niche (for more details see Fig. 2). This analysis was conducted in R with *ecospat* package and the R script is available in Suppl. material 2.

HOF curves

For zone A and B, 1325 randomly points were collected (25% of the total), thus obtaining a data vector of 1 (from zone A) and 0s (from zone B). The sampling procedure was repeated 50 times, thus obtaining for each time a data vector with 0s and 1s. Using these 50 data vectors, 50 HOF curves were generated (Oksanen and Minchin 2002). Basically, HOF curves are logistic regression models which represent species responses along environmental gradients, given a sample of suitable and unsuitable points (Fig. 2), the best model was selected using likelihood ratio tests or Akaike criteria (Ihaka and Gentleman 1996). HOF curves provide a set of parameters which describe different curve characteristics; one of them, the Outer Border defined by the gradient value where the response curve reaches exp (-2) relative to the highest estimated response value (Heegaard 2002). The latitude at which we obtained the Outer Border was considered an estimate of CNL predicted from global climatic niche. The bootstrap analysis was conducted in R, and R script is available in Suppl. material 3.

In summary, for each species, 50 HOF curves were generated, thus estimating 50 values for the Northern and 50 values for Southern CNL. To explore the variability of the estimates, a bootstrap distribution was used for Northern and Southern CNL, with 10.000 random resampling with replacement. If the RL falls within 95% confidence interval of bootstrap distribution of northern or southern CNL, the hypothesis is that RL – NL = 0 was accepted; otherwise, it was rejected.

Results

Acacia species (*A. dealbata and A. melanoxylon*) presents the broadest latitudinal range in Chile (Table 1); *A. dealbata* was the species with a higher number of presence records in the field (Table 1). On the other hand, *Trifolium suffocatum* was the species with the lowest latitudinal range and one of the species with lowest number of presence records in the field work in Chile (Table 1).

For the northern distribution of *Acacia dealbata* and *Cytisus striatus*, a matching between the observed and predicted north range limit was detected (Table 2), while for the rest of the species the observed north range limit was significantly lower than predicted (Table 2, Figs 3, 4). For the southern distribution of *Acacia dealbata* and *Cytisus striatus*, matching between observations and predictions was founded; for *Acacia melanoxylon*, observed RL was lower than expected, and for the rest of the species, the observed southern range limit was significantly higher than predictions (Table 3).

Discussion

The geographic position of RL was quite similar between the northern and southern range; at the northern range, in only two species (*Acacia dealbata* and *Cytisus striatus*), the RL was explained by climate. For the rest of species, the RL was explained by other factors such as dispersal limitation or negative biotic interactions. At the southern range, for *Acacia dealbata* and *Cytisus striatus*, the RL matched with climatic expectations as well; in one species (*Acacia melanoxylon*), RL was explained by facilitation and for the rest of species, RL was explained by negative biotic interactions and dispersal limitation.

For *A. dealbata and C. striatus*, climate explained RL either at the northern or at the southern border. This matching may indeed be attributed to their high ornamental value and widespread cultivation in parks, gardens and road borders, with strong anthropogenic subsidies in terms of resources and conditions (Van Kleunen et al. 2018; Beaury et al. 2023). This increased human-mediated dispersal and cultivation likely enhance their chances of expansion, allowing them to reach the maximum extent of their distribution without dispersal limitation. *A. dealbata* is regarded one of the most successful exotic trees in central Chile with significant impacts on native biodiversity (Fuentes-Ramírez et al. 2011). Our results give support to this invasive success. Firstly, its niche requirements match with prevailing climatic conditions; secondly, there is no dispersal limitation; thirdly it is a strong competitor over native plants due to allelopathic effects on the germination and growth of seed and seedlings (Aguilera et al. 2015), and a faster growth rate relative to native trees (Fuentes-Ramírez et al. 2011). However, there are some ecological constraints that can limit further expansion because it has resulted in becoming the most attacked exotic plant by herbivorous insects in invaded ranges which, in turn, can be used by biological control in different parts of the world (Wilson et al. 2011; Wilgen et al. 2023).

At the southern range, *Cytisus striatus* also matched RL with climatic niche. This result may be attributed to a low cold or freezing resistance of this species, as it has been documented in the northern hemisphere (Beans et al. 2012; Thomas and Moloney 2013; Winde et al. 2020).

Table 1. Number of local presence/absence records per species obtained from field work and global presence records obtained from different databases (see text above).

Species	Presence/absence points in central Chile				Latitudinal observed range and range size	Global presence points
	Presence coast	Absence coast	Presence valley	Absence valley		
A. dealbata	92	32	85	46	(-32, -43.1) (11.1°)	1123
A. melanoxylon	74	57	76	55	(-32, -42.9) (10.9°)	1300
C. striatus	31	100	27	104	(-33.1, -41.4) (8.3°)	731
T. monspessulana	51	80	38	93	(-32.9, -41.1) (8.2°)	245
U. europaeus	47	84	36	95	(-35.6, -43.0) (7.4°)	500
L. corniculatus	24	107	21	110	(-33.4, -43.1) (9.7°)	2079
T. suffocatum	0	131	2	129	(-34.6, -35) (0.4°)	682
V. villosa	1	130	3	128	(-32.9, -36.9) (4.0°)	1879

Table 2. Comparison between RL and CNL at the northern limit. CNL is represented by latitude values from 0.025 and 0.975 percentile (Q). For the northern limit the comparison has three possibilities: (i) RL = CNL, climate is enough to explain this limit; (ii) RL < CNL: competition and dispersal limitation explains this limit; (iii) RL > CNL: facilitation explains this limit.

Species	$Q_{0.025}$	$Q_{0.975}$	RL	RL - CNL	Hypothesis
A. dealbata	-30.37	-33.89	-32.0	RL = CNL	Climate
A. melanoxylon	-30.80	-30.92	- 32.0	RL < CNL	Competition/dispersal limitation
C. striatus	-32.18	-34.62	-33.1	RL = CNL	Climate
T. monspessulana	-31.06	-31.17	-33.1	RL < CNL	Competition/dispersal limitation
U. europaeus	-32.27	-32.40	-36.1	RL < CNL	Competition/dispersal limitation
L. corniculatus	-18.96	-19.21	-38.7	RL < CNL	Competition/dispersal limitation
T. suffocatum	-30.33	-31.76	-34.7	RL < CNL	Competition/dispersal limitation
V. villosa	-21.61	-25.60	-32.9	RL < CNL	Competition/dispersal limitation

Table 3. Comparison between CNL and RL observed at the southern limit. CNL is represented by latitude values from 0.025 and 0.975 percentile (Q). For the southern limit, the comparison has three possibilities: (i) RL = CNL: climate is enough to explain this limit; (ii) RL < CNL: facilitation explains this limit; (iii) RL > CNL: competition and dispersal limitation explain the limit.

Species	$Q_{0.025}$	$Q_{0.975}$	RL	RL - CNL	Hypothesis
A. dealbata	-43.32	-45.02	-43.1	RL = CNL	Climate
A. melanoxylon	-42.71	-42.82	-42.9	RL < CNL	Facilitation
C. striatus	-40.72	-42.29	-41.4	RL = CNL	Climate
T. monspessulana	-45.51	-45.61	-41.1	RL > CNL	Competition/dispersal limitation
U. europaeus	-49.08	-49.15	-43.0	RL > CNL	Competition/dispersal limitation
L. corniculatus	-47.60	-47.80	-43.1	RL > CNL	Competition/dispersal limitation
T. suffocatum	-38.33	-40.77	-35.0	RL > CNL	Competition/dispersal limitation
V. villosa	-53.64	-54.37	-36.9	RL > CNL	Competition/dispersal limitation

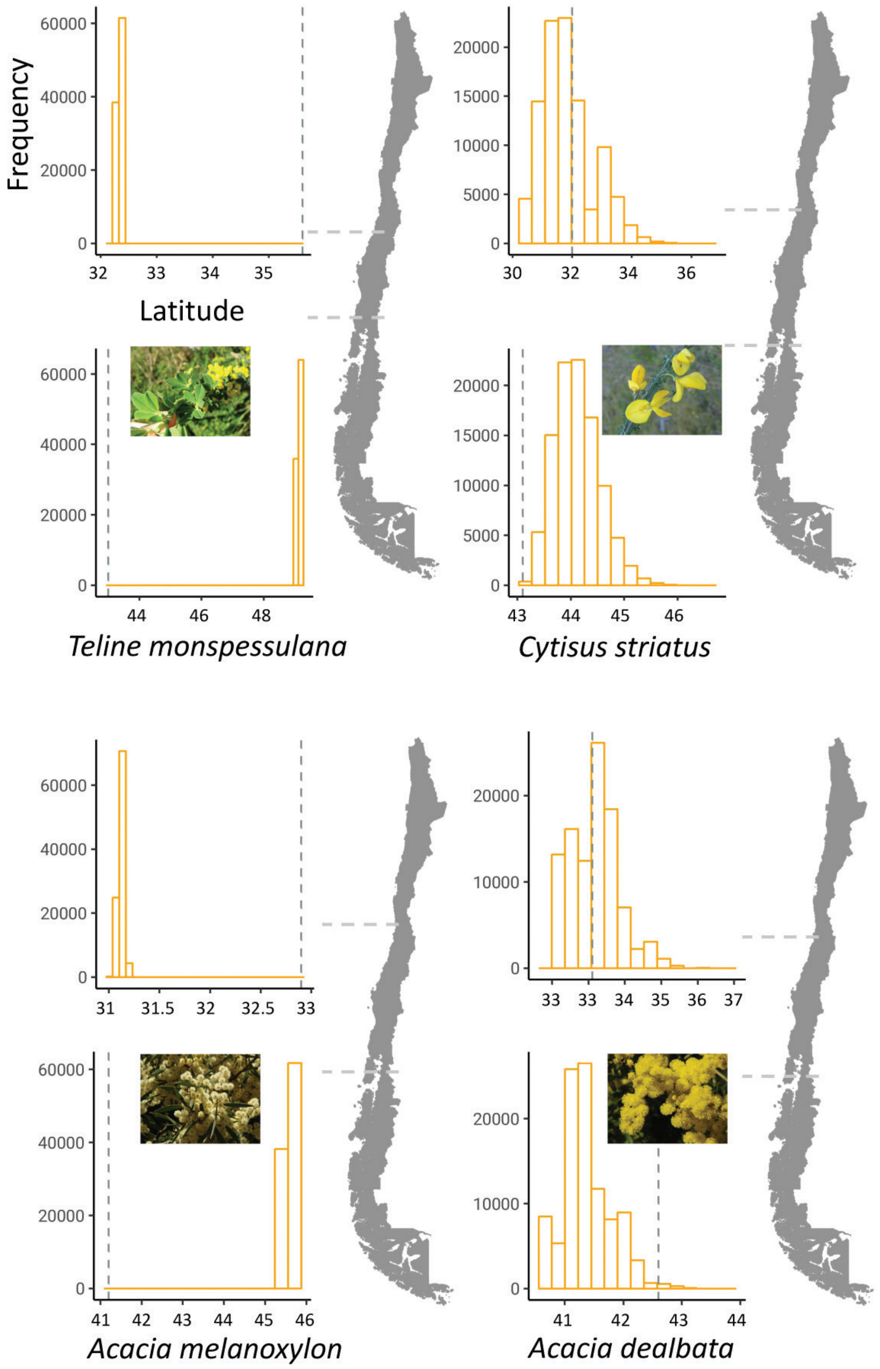

Figure 3. Bootstrap distribution of Northern and Southern CNL, obtained from global niche models, for *Teline monspessulana*, *Cytisus striatus*, *Acacia melanoxylon* and *Acacia dealbata*, central Chile.

At the northern range, the abiotic environments are relatively hostile to plant species. Under these conditions, plant-plant facilitation should be promoted, according to theory (Bertness and Callaway 1994; Lortie and Callaway 2006); however, we did not find evidence of such mechanism. We suggest that the mismatch detected between observation and expectation for six species (Table 1) can be attributed to dispersal limitations (low propagule pressure) due in part to a relatively low human settlement. The southern end of the climatic gradient in turn, hosts a high plant species diversity in Chile as well as an increase of the forest biomass (Bannister et al. 2012). This increased diversity may render these communities less invasible due to biotic resistance or competitive

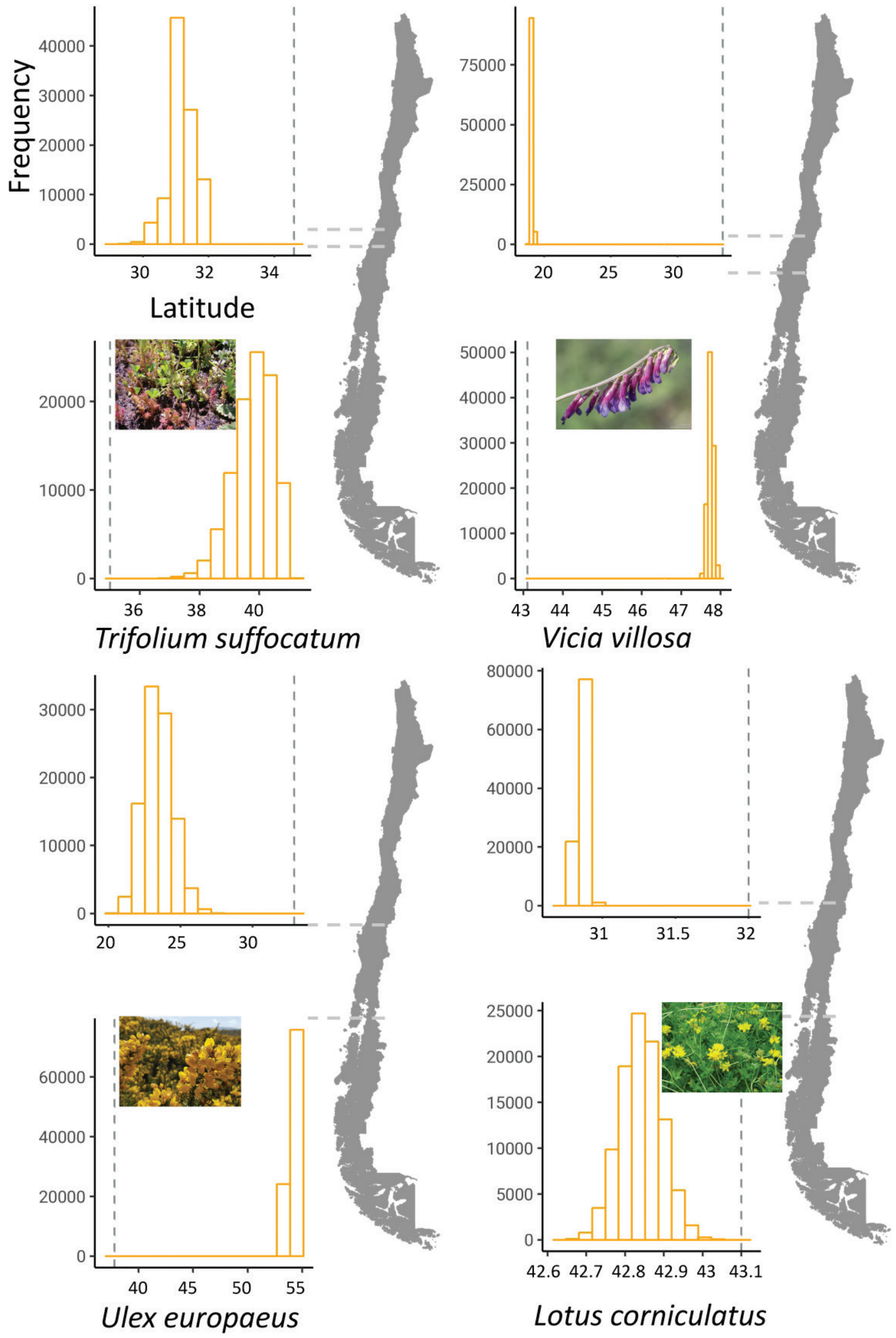

Figure 4. Bootstrap distribution of Northern and Southern CNL, obtained from global niche models, for *Trifolium suffocatum*, *Vicia villosa*, *Ulex europaeus* and *Lotus corniculatus*, central Chile.

mechanisms that limit the establishment of invasive species (Levine et al. 2004; Guo et al. 2023). Our results are consistent with Callaway's hypothesis (Bertness and Callaway 1994; Lortie and Callaway 2006), who suggests that less hostile environments, such as the southern border, may induce higher competitive pressure on exotic plants, thus resulting in constrained expansion of exotic plants further south. In summary, we propose that dispersal limitation may play an influential role at the northern border (lower latitude), while competition is more important at the southern border (higher latitude). Further field experiments are needed to test these biogeographic hypotheses.

In the southern range, *Acacia melanoxylon*, exhibited its RL beyond predictions from climatic niche. Mechanisms such as facilitation by human use, potential nurse species interactions, or local adaptation and expansion of tolerance ranges may explain these patterns. For example, a study by Turner et al. (2015) about invasive thistle (*C. diffusa*) suggests that the physiological tolerances of *C. diffusa* may have expanded in the invaded range. Invasive species tend to present adaptive plasticity and niche expansion (Moran and Alexander 2014; Pack et al. 2022). These hypotheses warrant further investigation through transplant experiments to elucidate the underlying mechanisms driving these distribution patterns.

The study of the causal factors that explain RL along environmental gradients has proven to be a fruitful research program linking biogeography, ecology and evolution (Holt and Keitt 2005; Sexton et al. 2009; Louthan et al. 2015). Most efforts have been addressed to designing proper field experiments to discern the microevolutionary and ecological factors which are responsible for such limits (Geber 2011; Hargreaves et al. 2014; Sexton and Dickman 2016); however less effort has been devoted to inferring RL from climatic niche using statistical techniques. The method applied in this study proposes a methodology to infer statistically, the expected range limits based on climatic niche requirements; this method establishes the geographic position species range limits, so allowing us to dispose with precision where to put transplants' experiments to test biogeographic hypotheses proposed by Hargreaves et al. (2014).

Conclusion

In conclusion, our study sheds light on the interplay between observed range limits and the global climatic niche for leguminous invasive plants in central Chile. While the climate-based limitation hypothesis is partially supported, with only two species showing concordance between niche and distribution at the northern and southern edge, our findings suggest that climatic conditions alone do not fully explain distribution patterns. This discrepancy between niche and distribution is particularly notable in areas with favorable climatic conditions, such as the southern extreme of the climatic gradient. Moreover, the idiosyncratic responses of species at both ends of the gradient highlight the importance of species-specific attributes and invasion processes that may influence distribution patterns. The complex interplay between climate, human activities, and ecological factors underscores the need for further research, particularly experimental studies, to validate and elucidate the underlying mechanisms shaping invasive plant distributions in mountainous regions like central Chile. Understanding these mechanisms is crucial for effective management and mitigation strategies aimed at controlling the spread and impact of invasive species in these ecosystems.

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

ROB conceptualized, EG and MD proposed the statistical design analysis, and AA data analysis; ROB, EG and MD contributed to the writing of the text.

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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

Global Occurrence records used per species, including date and source

Authors: Ramiro O. Bustamante, Aldo Alfaro, Estefany Goncalves, Milen Duarte

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.98.136183.suppl1

Supplementary material 2

Climaticniche in R

Authors: Ramiro O. Bustamante, Aldo Alfaro, Estefany Goncalves, Milen Duarte

Data type: txt

Link: https://doi.org/10.3897/neobiota.98.136183.suppl2

Supplementary material 3

HOF curves and bootstrat in R

Authors: Ramiro O. Bustamante, Aldo Alfaro, Estefany Goncalves, Milen Duarte

Data type: R file

Link: https://doi.org/10.3897/neobiota.98.136183.suppl3

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Research Article

Milkweed (*Asclepias syriaca*) invasion, forest-steppe fragment size and isolation jointly constrain arthropod communities and their functional traits

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Abstract

Habitat fragmentation has far-reaching negative impacts on the environment, resulting in biodiversity loss, soil quality degradation and alteration of water availability. In addition, fragmentation can disrupt ecological processes, potentially facilitating the establishment and spread of invasive plants, which can further harm native arthropod communities and alter their ecosystem dynamics. However, the exact nature of these impacts may vary depending on local conditions. We investigated the impact of fragmentation and milkweed invasion on invertebrate communities in sandy grasslands of forest-steppe habitats in Hungary. We selected 30 grasslands in forest-steppe fragments, varying in size (0.2 to 8.7 ha) and connectivity (Hanski's connectivity index: 0 to 705). We sampled ground-dwelling arthropods, mainly herbivores (true bugs) and predators (spiders), with pitfall traps and pollinators (wild bees) by direct observations along transects in invaded vs. non-invaded patches (min. of 500 m^2) of each fragment. We considered arthropod species' body size (all groups), dispersal ability and feeding (herbivores and predators) and nesting location and social habit (wild bee) traits in our analyses. In non-invaded patches, the number of monophagous herbivores showed an increasing trend, whereas in invaded fragments, there were more polyphagous individuals with increasing connectivity and fragment size. The dispersal ability of predators was lower as connectivity increased in non-invaded patches but higher in patches invaded by milkweed. We found more ground nesting bees in the invaded patches of small fragments than in large fragments, however, we did not find a significant effect in non-invaded patches. In summary, we often found interacting effects of the studied variables, fragmentation and invasions, generally modifying each other's effect by filtering for opposite trait levels. The primary objective of restoration projects should be restoring habitat of appropriate size and connectivity and eradicating invasive species while concurrently supporting the revival of native species and their ecological relationships. It is essential to employ adaptive management techniques, including continuous monitoring, to effectively tackle the interaction between fragmentation, invasion, and the preservation of biodiversity.

Key words: Biodiversity loss, connectivity, functional diversity, habitat degradation, landscape structure, sandy grassland

Introduction

Land-use changes, such as afforestation of grasslands, urbanisation and agricultural expansion, result in the fragmentation of natural habitats (Fischer and Lindenmayer 2007). Habitat fragmentation *per se* is independent of habitat loss, for a given amount of habitat, a more fragmented landscape has more, but smaller habitat patches (Fahrig 2017). Many small habitat patches seem to host more species than a few large patches of the same total area (Fahrig 2020). However, loss of habitat amount can negatively impact biodiversity, as it can limit the movement and gene flow of many species, increasing the risk of extinction and altering the species composition within habitat patches (Fahrig 2003; Tremlová and Münzbergová 2007). Conservation efforts may aim to restore habitat amount and connectivity between fragments to mitigate negative impacts (Wilson et al. 2016). Ensuring adequate habitat size and connectivity is crucial for maintaining biodiversity, promoting ecosystem health and supporting the resilience of species and ecosystems in the face of changing environmental conditions (Correa-Ayram et al. 2016).

Fragmentation can pave the way for non-native plant species to establish and spread, which may lead to adverse ecological impacts (Keller et al. 2008; Gutiérrez et al. 2014). Invasive plant species typically compete with native plants for resources like water, nutrients, light, and space or even pollinators, leading to reduced native vegetation diversity and a shift in the structure and composition of plant communities (Crooks 2002). These changes can have cascading effects on the whole ecosystem (Diez et al. 2010), including changes to the food web and the availability of animal nesting and foraging sites. For instance, invasive plants may not provide pollinators with the same quality or quantity of food resources as native plants (Bjerknes et al. 2007). This can lead to a reduction in pollinator densities, which can affect the reproductive success of native plants and the availability of fruits and seeds for their consumers.

Common milkweed (*Asclepias syriaca*) is native to North America. It is a herbaceous perennial plant that grows 30–180 cm tall and produces clusters of fragrant, pink or purple flowers in the summer. Common milkweed provides food and habitat for a wide variety of wildlife, including many specialists (Spaeth et al. 2022). Therefore, common milkweed has high nature conservation value in its native range; however, its invasiveness should be carefully monitored and managed outside of its native range (Zalai et al. 2017). In Europe, the common milkweed is considered an invasive species in several countries, and it is included in the list of invasive alien species of the European Union (EU list 2017). It was originally introduced to Europe in the 17th century as an ornamental plant and by beekeepers as a key food resource for honeybees due to its large quantities of nectar and pollen (Bukovinszky et al. 2014). Common milkweed was introduced to Hungary in the 18th century by beekeepers to increase food availability for their bees and improve the health and productivity of their hives. Milkweed became invasive and spread rapidly in the lowland areas of Central Europe (Bakacsy and Bagi 2020).

Milkweed has a negative effect on the native, habitat specialist plants. The most vulnerable areas to milkweed invasion are those where the sandy soils have been degraded, and habitat disturbance is already threatening the native vegetation of grasslands (e.g. disturbed by overgrazing, Bakacsy and Bagi 2020) and forest plantations (e.g. disturbed by intensive forestry, Ingle et al. 2019). Due to its aggressive growth habit, common milkweed can dominate and displace other plant species. Milkweed has a positive effect on habitat generalist and forest plants (Gallé et al. 2023).

Furthermore, it negatively affects grassland species, especially those with low competitive ability (Kelemen et al. 2016; Berki et al. 2023). Therefore, milkweed invasion has a general negative effect on the nature conservation value of invaded habitats. The presence of milkweed also alters vegetation structure, it increases the total cover and vegetation height and decreases bare ground cover (Gallé et al. 2023).

One of the most threatened habitat types by milkweed invasion is the forest steppes in southern Hungary (Bakacsy and Bagi 2020). Forest steppes are a mosaic of forest patches on grasslands (Fig. 1a). They form a distinct vegetation belt, a transition between closed forests and mostly treeless steppes in Eastern Europe and Asia (Bátori et al. 2018). Only small fragments of forest steppe remained in Southern Hungary, where fragment size and habitat connectivity are important drivers of arthropod diversity, especially in the forest-steppe grasslands (Gallé et al. 2022a), generally exerting a stronger effect on habitat specialist than generalist arthropods (Gallé et al. 2023).

Arthropods are essential parts of ecosystems, fulfilling multiple roles, such as herbivores, pollinators, predators, decomposers and prey for other organisms

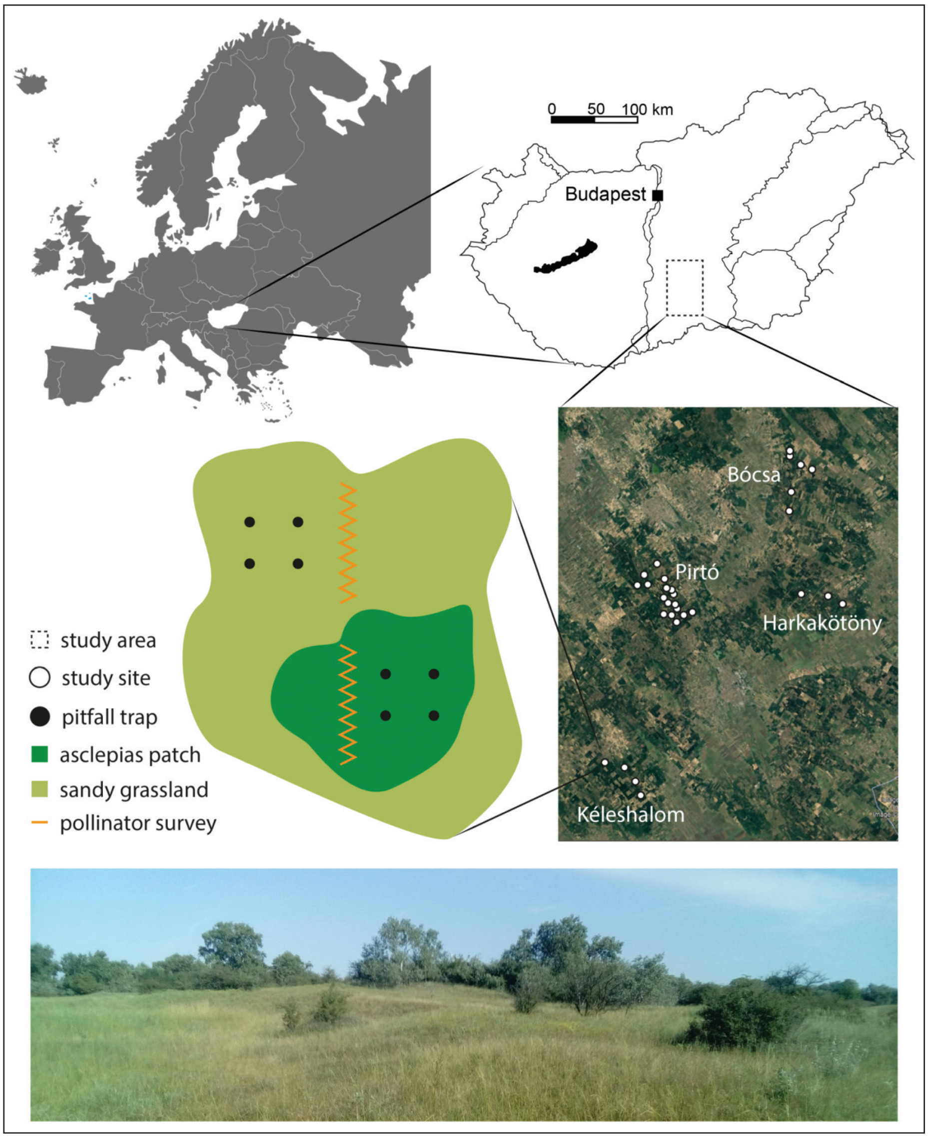

Figure 1. Map of study area showing the habitat fragments and the schematic figure of within-fragment sampling design.

(Schowalter 2022). Several factors affect arthropods' species and trait state composition. Focusing on functional traits can identify mechanisms that determine the impact of biodiversity on ecosystem processes (Spasojevic et al. 2018; Gallé and Batáry 2019). Identifying the drivers behind the trait state composition of arthropod communities can help us better understand their role in ecosystems and preserve their biodiversity (Haddad et al. 2008). The proliferation of an invasive plant, such as milkweed, may alter ecosystems, negatively impacting ground-dwelling arthropod populations (Gallé et al. 2015; Ingle et al. 2019). For example, Šeat et al. (2024) found that larger true bugs and species with generalist diets were associated with invasive plants in saline grasslands. Plant invasion may support web builders, reflecting vegetation composition and structural changes (Gomes et al. 2018). Connectivity and larger fragment size support large arthropod species, which can be expected to be good dispersers (Kormann et al. 2015, Korányi et al. 2023). Milkweed may affect wild bees positively, neutrally, and negatively (Szigeti et al. 2020; Kovács-Hostyánszki et al. 2022). The impact of common milkweed on wild bees depends on their traits, the local ecosystem and the abundance and distribution of other food and habitat resources (Gustafson et al. 2023).

In this study, we aimed to reveal the combined effects of milkweed invasion and habitat fragmentation on the functional diversity of three arthropod groups: herbivorous true bugs, wild bees and predatory spiders. We expected that (1) trait state and species composition differ between arthropod assemblages in invaded and non-invaded areas. We expected that varying connectivity and fragment size also shape trait composition: (2) Species dispersing over longer distances are able to populate less connected small fragments, whereas those with poor dispersal ability may be typical of well-connected large fragments; (3) Invasion homogenises food resources; thus species with a generalist diet are expected in invaded patches whereas feeding specialists are expected in areas that are close to natural.

Materials and methods

Study site and sampling design

We conducted our study in Southern Hungary (Fig. 1). This area is characterised by calcareous sandy alluvial soil with poor water retention capacity. The climate is continental, with 500–600 mm annual mean precipitation, and the mean annual temperature ranges from 10.2–10.8 °C (Török et al. 2003). The combined effect of climate and soil conditions allows the formation of forest-steppe vegetation community. Small patches of natural poplar forests (*Populus alba*), including juniper (*Juniperus communis*) and hawthorn bushes (*Crataegus monogyna*), are embedded in steppe grassland. The most common plant species of the steppe grasslands include *Festuca vaginata* and *Stipa borysthenica.* In the last century, vast natural forest steppes were converted into forest plantations of poplar and non-native pine trees and arable fields. The milkweed can spread easily in this region and form viable populations, especially in the grassland part of the remaining forest steppe fragments (Szitár et al. 2018).

We selected 30 forest-steppe fragments in a matrix of forest plantations along orthogonal gradients of fragment size and connectivity. Sampling sites were spatially clustered around four villages (Fig. 1). We conducted our samplings on the grasslands with similar vegetation characterised by dry bunchgrass steppes.

In each fragment, we established a pair of invaded vs. non-invaded (control) patches. Invaded patches had a milkweed stem density between 3–16/m^2. We selected invaded patches that covered at least 500 m^2, and sampling was done in the centre of the patch. Similarly, we sampled the centre of at least 500 m^2 non-invaded patches. Herbaceous vegetation was somewhat higher and denser in invaded patches than in non-invaded patches (Gallé et al. 2023). We avoided the edges of forest-steppe fragments, hence all sampling was done at least 40 m from the edges. We measured the size of the fragments using Quantum GIS 3.6.1 software and satellite images (Quantum GIS Development Team 2019). The size of the fragments varied between 0.2 and 8.7 ha and the distance to the nearest fragment varied between 15 m and 570 m. We also calculated the connectivity of fragments using Hanski's connectivity index within a buffer of 500 m around all fragments (Hanski et al. 2000), which seems to be appropriate in studying forest-steppe arthropods of the region (Gallé et al. 2022 a,b). Connectivity values ranged between 0 (isolated) and 705 (well-connected).

We used pitfall traps equipped with a funnel and a roof to collect ground-dwelling arthropods (Császár et al. 2018). We installed four traps at each sampling patch five meters from each other, in a square (Fig. 1). Traps were deployed for two weeks (9–27 July 2021) and we emptied the traps after one week. We identified true bugs and spiders at the species level. The data from the four traps was pooled for each patch.

We surveyed flower-visiting insects (wild bees belonging to the Hymenoptera order except for honeybees) using the transect method (50 m-long zig-zag transect surveyed for 15 minutes) (Fig. 1). We excluded honeybees as their presence was dependent on the presence of farmers' hives. We recorded all wild bees at a distance of 2.5 m to the right and left sides of the transect. Sampling was carried out by the same person (ET). All wild bees that could not be identified in the field were collected with standard entomological nets, stored in 70% ethanol and identified with a stereomicroscope. We performed two sampling rounds, the first at the beginning of the milkweed flowering period (10.06.-15.06.2021) and the second at the peak of the flowering period (28.06.-02.07.2021). Voucher specimens were placed in the arthropod collection of the HUN-REN Centre for Ecological Research (spiders and hymenopterans) and the University of Szeged (true bugs).

Arthropod functional traits

Body size of all species was given as a continuous variable in mm (mean body length averaged over males and females). For the other traits, we used ordinal categories. For true bugs, we used the wing length as a proxy for dispersal ability (0 – brachypterous, 0.25 – predominantly brachypterous, 0.5 – equally brachypterous and macropterous, 0.75 – predominantly macropterous, 1 – macropterous), and their diet range (0 – monophagous, 0.5 – oligophagous, 1 – polyphagous). For wild bees, social habit (0 – solitary, 0.5 – subsocial, 1 social) trait and nesting height (0 – in the soil, 0.5 – on herbaceous vegetation, 1 – tree trunk) was used. In the case of spiders, we took into account their dispersal ability, which was indicated by the ballooning trait (0 – the species is not likely to balloon, 0.5 – at least a single species is known to balloon in the genus, 1 – the species balloon) and their hunting strategy (0 – active ground hunters, 0.5 – ambush hunters on vegetation, 1 – web-builders).

In order to moderate the weight of the large values, trait values ranged between 0 and 1 (Suppl. material 1). Trait values were collected from the following literature: Panizzi and Grazia (2015), Wachmann et al. (2008), Bees, Wasps and Ants Recording Society (BWARS 2021), Witt (1998), Falk (2015), Cardoso et al. (2011), Bell et al. (2005), Blandenier (2009) and Nentwig et al. (2017).

Data analyses

We calculated the community-weighted means (CWM) for each trait, using the averages of trait values weighted by the relative abundances of each species at each sampling site. We applied linear mixed-effects models to investigate the effect of invasion, fragment size, connectivity and their two-way interactions on arthropod communities (lmer function in package lme4, Bates 2010). We log-transformed fragment size values before the analyses. We included the fragment ID nested in the nearest village (corresponding to the spatial clusters of fragments) in the model as a random effect to consider potential spatial autocorrelation. We used diagnostic figures (Q-Q plots, residuals vs. fitted values) to check whether the model assumptions were met. We transformed response variable data if we detected deviation from model assumptions (see Table 1, for details). Furthermore, we used Cook's distance (Cook 1979) to measure an observation's influence on the

Table 1. Summary table for linear mixed-effects model results on true bug, wild bee and spider communities showing model parameter estimates of each variable ± 95% confidence intervals. Significant p-values at $p < 0.05$ are indicated in bold. "Invasion" refers to the presence of invasive milkweed; we kept non-invaded as the reference factor level for "Invasion".

Herbivores	**Size[1]**	**Dispersal**	**Diet[2]**
Invasion	**-0.340 ± 0.257**	0.129 ± 0.156	**-0.435 ± 0.224**
Fragment size	-0.205 ± 0.534	0.112 ± 0.292	-0.102 ± 0.407
Connectivity	-0.334 ± 0.504	**0.291 ± 0.263**	0.099 ± 0.386
Invasion × Fragment size	-0.155 ± 0.361	0.005 ± 0.219	**0.479 ± 0.315**
Invasion × Connectivity	**0.425 ± 0.348**	-0.141 ± 0.210	**0.400 ± 0.304**
Fragment size × Connectivity	0.150 ± 0.935	-0.307 ± 0.474	-0.533 ± 0.700
Bees	**Size[3]**	**Nesting[4]**	**Social habit**
Invasion	0.041 ± 0.372	**-0.283 ± 0.262**	-0.017 ± 0.410
Fragment size	-0.311 ± 0.778	-0.241 ± 0.431	-0.101 ± 0.676
Connectivity	-0.037 ± 0.742	-0.242 ± 0.407	0.324 ± 0.639
Invasion × Fragment size	0.309 ± 0.522	**0.443 ± 0.368**	0.388 ± 0.577
Invasion × Connectivity	0.005 ± 0.504	0.252 ± 0.355	-0.131 ± 0.294
Fragment size × Connectivity	0.358 ± 1.379	0.367 ± 0.713	-0.133 ± 0.861
Predators	**Size[5]**	**Dispersal**	**Hunting[4]**
Invasion	-0.268 ± 0.264	-0.096 ± 0.132	0.060 ± 0.096
Fragment size	0.272 ± 0.469	-0.208 ± 0.274	0.073 ± 0.158
Connectivity	0.339 ± 0.431	**-0.320 ± 0.259**	-0.037 ± 0.149
Invasion × Fragment size	0.154 ± 0.388	-0.015 ± 0.186	-0.088 ± 0.135
Invasion × Connectivity	0.238 ± 0.375	**0.227 ± 0.181**	-0.020 ± 0.130
Fragment size × Connectivity	-0.796 ± 0.761	0.432 ± 0.509	0.083 ± 0.264

[1] variable was inverse transformed and ranged between 0 and 1.
[2] variable was qubic transformed and ranged between 0 and 1.
[3] variable was box-cox ($\lambda = 2.1$) transformed and ranged between 0 and 1.
[4] model was refitted with zero-inflated generalised linear model.
[5] variable was log-transformed and ranged between 0 and 1.

coefficients' estimation. We checked the data for influential points with Cook's distance and did not detect any. We employed PERMANOVA using Bray-Curtis distance measure to examine the multivariate response of arthropod communities to factors such as fragment size, connectivity, and the presence of milkweed (adonis2 function in vegan package Oksanen et al. 2019). If the PERMANOVA detected significant differences between arthropod communities of invaded and non-invaded habitat patches, we applied the indicator value analysis to identify characteristic species. We calculated the indicator value (IndVal) based on the relative frequency and average abundance of the species we sampled (Dufrêne and Legendre 1997, indval function in package labdsv, Roberts 2019). The statistical significances of the indicator values were estimated by 9999 random permutations of fragments across groups. We visualised the community composition of arthropods using non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity measure and 100 random starts with the vegan package (Oksanen et al. 2019). Prior to ordination, we transformed the species abundance data into relative species values by applying a Hellinger transformation. This transformation aimed to decrease the influence of species with high abundances (Legendre and Gallagher 2001). We used the R Studio software and the R statistical environment (R Core Team 2022) for all analyses.

Results

We collected 2797 true bug individuals belonging to 58 species, 854 wild bees belonging to 30 species and 2544 spiders representing 65 species (Suppl. material 1). The CWM of true-bugs body size increased with increasing connectivity in invaded patches but decreased in non-invaded ones. (Table 1, Fig. 2A). Connectivity had a significant positive effect on the wing length of true bugs, as there were more individuals with developed wings in well-connected fragments than in poorly connected fragments (Table 1, Fig. 2B). We found a similar pattern for fragment size and connectivity effect on herbivore diet as it was influenced by both fragment size and connectivity in interaction with invasion. The diet of herbivorous true bugs became more specialised (i.e., the community shifted from polyphagous toward monophagous individuals) in non-invaded patches and increased in invaded fragments with increasing connectivity and fragment size (Table 1, Fig. 2C, D).

Bee individuals with higher nesting locations were more common in non-invaded patches. We also detected an interaction effect of invasion and fragment size on the CWMs of wild bees' nesting strategy. We found more ground-nesting wild bees in non-invaded patches in small than large fragments (Table 1, Fig. 3E).

The ballooning propensity, the proxy for the dispersal ability of spiders decreased (i.e. the number of poorly dispersing individuals increased) in non-invaded patches as connectivity increased. In contrast, in patches invaded by milkweed, CWM of dispersal ability values increased with increasing connectivity (Table 1, Fig. 3F).

We found significant differences in the wild bee and predator community composition of the invaded and the non-invaded patches (Table 2). We detected a single indicator wild bee species, namely *Bombus terrestris*, in the invaded habitats (Fig. 3A). In the case of predators, we found one indicator species, the salticid *Pellenes nigrociliatus*, for the non-invaded and three gnaphosid species, *Haplodrassus bohemicus*, *Zelotes exigus*, *Zelotes segrex* for the invaded fragments (Fig. 3B). No differences in community composition were observed for herbivores (Table 2).

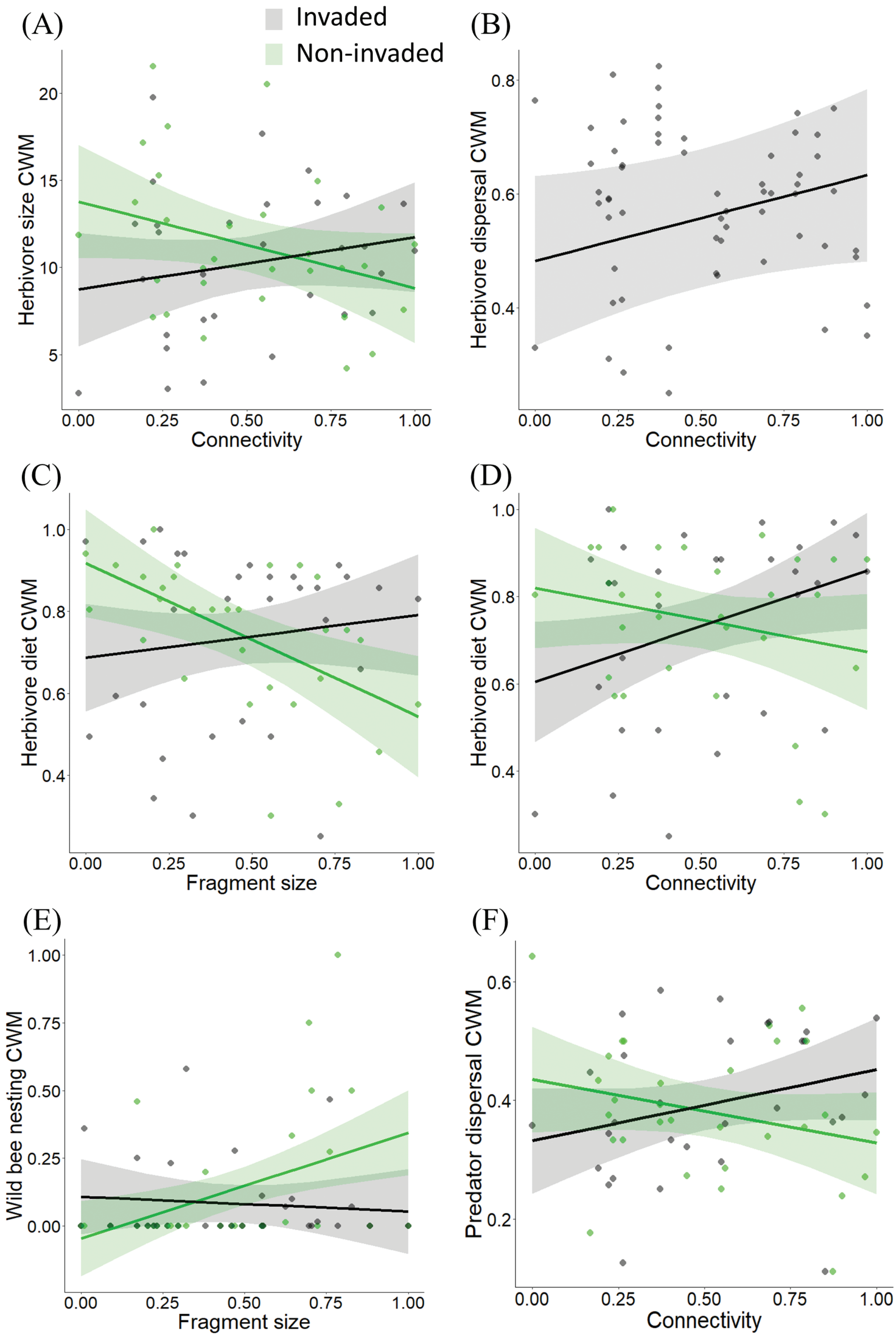

Figure 2. Effects of invasion and landscape variables on arthropod functional trait CWMs (community weighted mean). These are abundance-weighted averages of indices ranging from 0-1 (see Methods) **A** interacting effect of milkweed invasion and connectivity on herbivore size CWM **B** connectivity affect dispersal CWM of herbivores **C** invasion and fragment size effect on herbivore diet CWM **D** the interacting effect of invasion and on the diet CWMs of herbivores **E** interacting effect of invasion and fragment size on wild bee species nest height CWM **F** interacting effect of invasion and fragment size on spider dispersal CWM. Ranged values of connectivity and log-transformed fragment size are plotted. Grey dots show invaded sites and green dots show non-invaded sites. We show fitted regression lines with 95% confidence intervals.

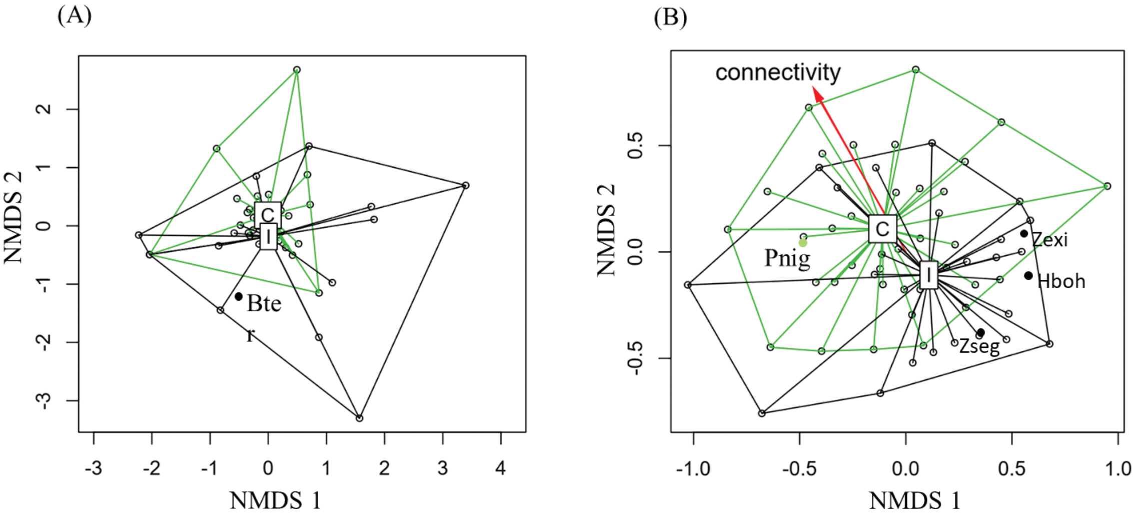

Figure 3. Non-metric multidimensional scaling ordination (NMDS) of **A** wild bee (stress = 0.073), and **B** predator (stress = 0.260) community composition. Sites are indicated with open circles. Red arrow indicates the significant continuous variable, and convex hulls indicate the habitat (C: control (non-invaded), I: invaded). Indicator species of invaded areas are indicated with black dots, and indicator species of non-invaded areas are green dots. Bter: *Bombus terrestris* (Apidae), Pnig: *Pellenes nigrociliatus* (Salticidae), Zexi: *Zelotes exigus* (Gnaphosidae), Hboh: *Haplodrassus bohemicus* (Gnaphosidae) and Zseg: *Zelotes segrex* (Gnaphosidae).

Table 2. PERMANOVA results for studied arthropod communities. Significant p-values are indicated in bold ($p < 0.05$).

	Herbivore			Wild bees			Predator		
	R^2	F	*P*	R^2	F	*p*	R^2	F	*p*
Invasion	0.030	1.788	0.083	0.035	2.097	**0.033**	0.034	2.108	**0.011**
Connectivity	0.016	0.970	0.456	0.009	0.548	0.861	0.032	1.973	**0.017**
Fragment size	0.004	0.293	0.978	0.020	1.217	0.256	0.019	1.196	0.260

Discussion

We studied the effect of invasive milkweed, fragment size, and connectivity on different arthropod communities inhabiting a threatened habitat type, the forest steppe. We found an interacting effect of the presence of the invasive species, connectivity and fragment size affected on most arthropod traits. Specifically, our hypothesis (1) was supported: invasion or its interaction with fragment size or connectivity affected the traits of all arthropods studied. Hypothesis (2) was also supported: the presence of the invasive plant shaped the community composition of wild bees and spiders. Hypothesis (3) was supported for true bugs in well-connected fragments: we found more polyphagous herbivores in invaded sites than in non-invaded sites.

Corridors or interconnected patches facilitate large individuals' movement, increasing the likelihood of their presence, but this effect was not attributed to a single or few species. Large herbivores typically require ample food resources to sustain their larger body size (Tscharntke et al. 2002). Herbivore body size increased with higher connectivity in non-invaded but decreased in invaded patches. This suggests that smaller herbivorous insects might be more common in well-connected habitats. But the presence of invasive plants filters for smaller species and disadvantaging the larger species in poorly connected habitats.The collected true bug species mainly rely on native plants, generally avoiding milkweed, as this plant is poisonous to many herbivores. Only a few European true bug species feed on the milkweed, from the genus *Lygaeus*. Indeed, we collected more *Lygaeus simulans* individuals in invaded (34 individuals, 77%) than in non-invaded areas (10 individuals, 22%). Native plants of the forest-steppe grasslands can be more accessible and readily available in non-invaded patches, promoting the occurrence of larger true bug species. If the preferred host plants of a true bug species are negatively affected by invasive plants, this can lead to a decline in the abundance of that true bug species (Crooks 2002). This may occur despite increasing habitat connectivity.

In line with our expectations, large and well-connected fragments supported feeding specialists if fragments were non-invaded (e.g. Fischer and Lindenmayer 2007). The herbivore community shifted towards species with specialist diets in non-invaded patches, but in invaded patches, the generalist species were more frequent with increasing connectivity and fragment size. This suggests that invasive plants can affect herbivores' feeding specialisation, resulting in more generalists in large and connected fragments. The presence of invasive milkweed can have a negative impact on herbivores both directly and indirectly (Gallé et al. 2023; Korányi et al. 2023). Herbivores encounter invasive plants with chemical compositions that differ significantly from those of native plants (Xiao et al. 2020). Native herbivores are not adapted, or are poorly adapted, to feed on invasive plants. The indirect effect of milkweed on feeding specialisation might have been established through the effect of the invasive plant on native vegetation. Invasive plants negatively affect the native plant communities (Vilà et al. 2011). Consequently, they may reduce the access of specialist herbivores to their native host plants. Milkweed affects the species composition of native vegetation by supporting more generalist plant species, which provide a more diverse diet for generalist true bugs (Gallé et al. 2023). Moreover, milkweed can provide shade through its canopy and litter, potentially alleviating unfavourable abiotic (microclimate) conditions for the germination of specialist plant species (Szitár et al. 2018). Furthermore, the large stems of milkweed change the habitat structure of sandy grasslands, presumably reducing the availability of suitable oviposition sites and compromising herbivores' survival and reproductive success (Tallamy and Shropshire 2009). The generalist plants in invaded patches might formed a suitable habitat for generalist herbivores; therefore, connectivity and fragment size supported their high abundance.

The availability of suitable nesting sites is crucial for solitary wild bees, making them highly vulnerable to unfavourable environmental factors and the impacts of human-induced changes (Harmon-Threatt 2020). Milkweed was the highest herbaceous plant species in our study, producing large quantities of relatively high stems. Above-ground nesting bees build their nests in holes in plant stems or dead wood (Bihaly et al. 2021). The increased ratio of wild bees nesting on higher vegetation in non-invaded patches means that large milkweed stems did not offer more suitable nesting sites for above-ground nesters. Additionally, the large fragments support a larger population of vegetation-nesters, presumably by offering more nesting sites and more available food resources. Minimising the distance wild bees have to cover between the food resource and nesting location during foraging is essential to allocating energy for their reproduction. Non-invaded patches bear lower average vegetation heights and more bare soil surfaces, which create ideal conditions for ground-nesting species (Harmon-Threatt 2020). The majority of recorded bee species nest in the soil and spend much of their life cycle underground. Their preferences may differ concerning soil type, texture, compaction, moisture, and temperature (Antoine and Forrest 2021). Lower soil moisture and lower ground surface temperatures caused by milkweed invasion (Gallé et al. 2023) could potentially limit ground-nesting species (Pellaton et al. 2024), however ground-nesting bees generally dominated the communities of forest-steppe fragments. Large and well-connected, flower-rich forest-steppe fragments are beneficial for ground-nesting bees (Török et al. 2022). Further, the impact of milkweed invasion on different floral visitor groups may vary, as their diversity is probably influenced by the presence and variety of native flowering resources (Kovács-Hostyánszki et al. 2022). However, it did not affect the size and social behaviour of wild bees.

Well-connected fragments are often viewed as higher-quality habitats with more specialist species. They support a high species richness and abundance of arthropods (Hanski 1998), which are potential food items for predators, provide suitable conditions and high-quality habitats for many spiders including poorly dispersing species (Gallé et al. 2022a). In sandy grasslands, vegetation structure is also among the most important parameters that affects spiders (Carvalho et al. 2011). Specifically, vegetation with complex structures supports many spider species (Gallé et al. 2010). Milkweed increases vegetation complexity, and, therefore might have an indirect positive effect on spiders and change trait state composition of the community. We found that predator dispersal ability decreased with connectivity in non-invaded patches, but increased in patches invaded by milkweed.

Spiders with good dispersal ability can move between fragments more efficiently in landscapes with well-connected fragments, facilitating gene flow and maintaining stable populations (Gallé et al. 2022b). In contrast, limited connectivity hinders the movement of spiders with poor dispersal ability, as they may not be able to traverse the vast distances between patches effectively (Gallé et al. 2022c). Consequently, these spiders are more likely to remain confined within their habitat patch and have limited opportunities to disperse or colonise new areas.

The differences in community composition between invaded and non-invaded patches of wild bees and predators could be because invasive species can alter the availability and quality of resources (Bartomeus et al. 2008) and habitats for these groups (Pyšek and Richardson 2007), leading to changes in their community composition. Indicator species were the most sensitive to the differences in the conditions between invaded and non-invaded habitats. We found a single indicator wild bee species for invaded patches, *B. terrestris*, an abundant ground-nesting bumblebee species in Europe. Our results showed that milkweed flowers attract *B. terrestris*. This highly mobile insect can fly over 1 km for flower resources (Osborne et al. 2008). Due to its high foraging range and sufficient tongue length to access the nectar of milkweed flowers, it is one of the few polylectic bee species that can utilise the milkweed nectar (Kephart and Theiss 2004). In invaded patches, the large quantity of nectar provided by milkweed might distract pollinating insects that are capable of utilising milkweed nectar away from native plants and affecting their pollination services of native plants (Goulson et al. 2010), with a potential negative long-term impact on them (Kovács-Hostyánszki et al. 2022).

In invaded habitats, alterations in the availability of potential spider prey associated with milkweed invasions may have cascading effects on spider populations. In our study, we found significant differences in predator community composition between invaded and non-invaded patches, with a single indicator species (*P. nigrociliatus*) preferring non-invaded vegetation and avoiding invaded patches. Three indicator species of invaded patches (*H. bohemicus*, *Z. exigus*, *Z. segrex*) were xerophilous species. The presence of milkweed provides drier and more favourable conditions for these species (Gallé et al. 2023).

Our study highlights the complex interactions between fragmentation and invasion on ecological traits of herbivores, wild bees, and predators. Our research revealed that fragmentation and invasions interacted, typically altering their respective impacts by selectively favouring opposite trait levels. Our results suggest that invasive species can significantly impact the traits of arthropods and that larger and better-connected fragments may not necessarily provide better habitats. Therefore, we suggest that restoration projects should aim to restore habitats and their connectivity and eliminate invasive species from natural habitats. This may involve targeted removal or control of invasive species while promoting the recovery of native species and their ecological interactions. Future research should focus on the effects of milkweed removal on plants and arthropods, including detailed food web analyses, assessing the direct and indirect effects of management (e.g. via altered habitat parameters) and providing an opportunity to identify species that are sensitive to or supported by management interventions. Given the complexity of interactions between fragmentation (size and connectivity) and invasion, adaptive management approaches incorporating ongoing monitoring and evaluation are crucial.

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

RG, PB: Conceptualization, RG: Methodology, Software, Validation, Formal analysis, RG, ARS, ET, LT, DK, AT, G-SN: Investigation, RG, PB: Resources, Funding Acquisition, RG, G-SN: Data Curation, RG, ARS: Writing – Original draft, RG, ARS, ET, LT, DK, AT, G-SN, PB: Writing – Review and Editing, G-SN: Visualization.

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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

List of species and trait values

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Data type: docx

Explanation note: The trait values of species are given. For true bugs, we used the wing length as a proxy for dispersal ability (0 – brachypterous, 0.25 – predominantly brachypterous, 0.5 – equally brachypterous and macropterous, 0.75 – predominantly macropterous, 1 – macropterous), and their diet range (0 – monophagous, 0.5 – oligophagous, 1 – polyphagous). For wild bees, social habit (0 – solitary, 0.5 – subsocial, 1 social) trait and nesting height (0 – in the soil, 0.5 – on vegetation, 1 – tree trunk) was used. In the case of spiders, we took into account their dispersal ability, which was indicated by the ballooning trait (0 – the species is not likely to balloon, 0.5 – at least a single species is known to balloon in the genus, 1 – the species balloon) and their hunting strategy (0 – active hunters, 1 – web-builders).

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Research Article

Intra- and interspecific pollen morphology variation of invasive *Reynoutria* taxa (Polygonaceae) in their response to different habitat conditions

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Abstract

Although understanding the relationship between the reproductive mode and mass-dispersal potential of plants is crucial for studying invasion phenomena, the morphological features of invasive species' pollen are not well understood. This study examined the pollen morphology and variability of three *Reynoutria* (knotweed) taxa (*R. japonica*, *R. sachalinensis*, *R. ×bohemica*) invasive in Europe, and their reaction to different habitat conditions within seven distinguished habitat types. The pollen was sourced from 95 sites from the taxa's invasive range in Central Europe. In total, 2850 pollen grains were measured and analysed for 11 quantitative and qualitative features. The pollen of *R. sachalinensis* was distinguished from that of the other two taxa (reticulate perforate) based on its rugulate and fossulate perforate exine ornamentation. The pollen's response to various habitat conditions, which was most marked in *R. ×bohemica*, was reflected by pollen size and exine thickness. Our research indicates that pollen availability is not a limiting factor for the sexual reproduction of knotweeds in Central Europe, including *Reynoutria japonica*, long considered male sterile. The observed presence of male-fertile specimens of *R. japonica* may enhance the efficiency of generative reproduction in this species throughout its invaded ranges. This finding should be considered when planning actions to control the population of these transformer plant species.

Key words: *Fallopia*, habitats, invasive plant species, pollen morphology, pollen variability, *Reynoutria*, SEM

Introduction

Taxa of the knotweed genus *Reynoutria* are currently considered to be among the most aggressive plant invaders in both Europe and North America, and have become one of the most intractable weeds to manage across the globe (Child and Wade 2000; Weber 2017; Cottet et al. 2020; Bzdęga et al. 2021; Hocking et al. 2023; Švec et al. 2024). Taxonomically, these robust Asian woody-stemmed herbaceous perennial plants belong to the subtribe Reynoutriineae in the Polygonaceae family of knotweeds from the order Polygonales, which includes, depending on the taxonomic approach, 40 (Balogh 2008) to 49 genera (Sanchez et al. 2011; Schuster et al. 2011). ‘Knotweed’ is a collective term used to refer to representatives of the genus *Reynoutria* Houtt. (syn. *Fallopia* Adans.) (= *Fallopia* sect. *Reynoutria* (Houtt.) Ronse De Craene). These strongly rhizomatous perennials include two species, *Reynoutria japonica* Houtt. (*Fallopia japonica* (Houtt.) Ronse Decr.) and *R. sachalinensis* (F. Schmidt) Nakai (*F. sachalinensis* (F. Schmidt) Ronse Decr.), and their hybrids, *R.* ×*bohemica* Chrtek & Chrtková (*Fallopia* ×*bohemica* (Chrtek & Chrtková) J.P. Bailey), as well as any backcrosses and hybrids resulting from crosses with other related species, including *F. baldschuanica* (Regel) Holub (sect. Sarmentosae (I. Grintz.) Holub) (Bailey and Wisskirchen 2006; Bailey et al. 2007, 2009; Hocking et al. 2019; Stace 2019; Hodálová et al. 2022).

Reynoutria japonica and *R. sachalinensis* are native to East Asia (Shaw 2008, 2013). The native range of *R. japonica* extends from the northernmost parts of the Sakhalin and the Kuril Islands, through Japan, Korea and Taiwan to Vietnam in the south (Bailey 2003; Balogh 2008; Alberternst and Böhmer 2011). The area of natural occurrence of *R. sachalinensis* is much smaller, covering Sakhalin, the southern Kurils, Japan (northern Hokkaido and part of Honshu) and Ullyng Island (Bailey and Conolly 2000; Balogh 2008). In recent decades, *R. japonica* and *R. sachalinensis* have spread outside of their native distribution range into Europe, North America, Australia and New Zealand (Gibbs et al. 1987; Beerling et al. 1994; Sukopp and Starfinger 1995; Owen 1996; Bailey and Wisskirchen 2006; Barney et al. 2006; Bailey et al. 2007, 2009; Shaw 2008, 2013; Tokarska-Guzik et al. 2017). In addition, *R. japonica* occurs in South America (Chile) (Saldaña et al. 2009) and *R. sachalinensis* was found in South Africa (Shaw 2013). In Europe, these species were introduced in the 19th century as decorative garden plants and spread rapidly into natural habitats (Bailey and Conolly 2000; Bailey 2013). In 1983, in what was then Czechoslovakia, a hybrid of two species, *Reynoutria* ×*bohemica*, was first observed and described (Chrtek and Chrtková 1983). After this publication, sites of the hybrid were found in other European countries (Bailey et al. 1995; Keil and Alberternst 1995; Fojcik and Tokarska-Guzik 2000; Balogh 2008), and its presence was also confirmed in the area of natural occurrence of knotweed in Japan (Bailey 2003).

In their native ranges, *R. japonica* and *R. sachalinensis* propagate both sexually and vegetatively (Shaw 2008, 2013). In invaded ranges, it has been assumed that all knotweed taxa mainly reproduce vegetatively and disperse clonally by the growth/regeneration of rhizomes and shoots, and by the fragmentation of these organs (e.g. Brock et al. 1995; Shaw and Seiger 2002; Bailey et al. 2009; Alberternst and Böhmer 2011; Strgulc Krajšek and Dolenc Koce 2015). Despite the development of viable seeds (Forman and Kesseli 2003; Strgulc Krajšek and Dolenc Koce 2015) and the normal growth and development of knotweed seedlings in laboratory conditions (Bailey 1994; Forman and Kesseli 2003; Engler et al. 2011; Kadlecová et al. 2024), in the wild successful sexual reproduction of these taxa was considered rather rare (Pyšek et al. 2003; Tiébré et al. 2007; Bailey et al. 2009; Engler et al. 2011). However, according to some authors, in the case of *R.* ×*bohemica*, sexual reproduction is the main factor determining its invasive character (Buhk and Thielsch 2015; Strgulc Krajšek and Dolenc Koce 2015; Tokarska-Guzik et al. 2017). Hybrids are most often produced from seeds developing on *R. japonica* shoots. This is the result of pollination of the ‘pistillate’ flowers with pollen from *R. sachalinensis* flowers or related species (Bailey 2013; Strgulc Krajšek and Dolenc Koce 2015; Tokarska-Guzik et al. 2017).

Knotweeds are described as dioecious plants, characterized by the presence of two groups of individuals in one species: one produces hermaphroditic flowers, and the other produces female flowers (male sterile, so-called gynodiecia) (Bailey 1994; Bailey et al. 1996; Forman and Kesseli 2003; Alberternst and Böhmer 2011). The breeding system of *R. japonica* in Europe has been described as dioecious and gynodioecious (Bailey 1994), but all plants were found to be functionally female (male-sterile) (Bailey 1994; Beerling et al. 1994; Hollingsworth and Bailey 2000). This caused European populations of this plant to be considered as a genetically uniform single female clone (Bailey et al. 2009). However, there are some indications that due to the different histories and dynamics of *R. japonica* invasions in Western and Central Europe and North America, invasive populations of these plants may have other characteristics (Bzdęga et al. 2012). Although genetic analyses confirmed the lack of genetic diversity of the *R. japonica* population in Britain (Hollingsworth and Bailey 2000), such variation was found in populations in North America (Grimsby et al. 2007) and between individuals of *R. japonica* in Central Europe (Bzdęga et al. 2012; Mereďa et al. 2023). This diversity may be the result of episodes of sexual reproduction (Bzdęga et al. 2012), i.e. the occurrence of male flowers with pollen.

In their native range, *Reynoutria japonica* and *R. sachalinensis* show high tolerance to environmental factors and have similar soil requirements and preferences for soil reaction (Shaw 2008, 2013). Both species most often occur in open and sunny habitats and, as pioneer species, often become established on the slopes of volcanoes. *Reynoutria sachalinensis* grows on mountain landslides and areas along ravines and mountain streams, encroaches on seaside cliffs, and is also found on the edges of forests, whereas *R. japonica* prefers open and humid areas, where, like *R. sachalinensis*, it usually grows on sunny slopes of hills and forest edges; more often, however, it is found on the banks of ditches and roadsides (Bailey and Conolly 2000; Bailey 2003).

To date, the pollen morphology of species belonging to several genera from the family Polygonaceae has been described. The most frequently described genera among them are *Rumex* and *Polygonum*, then, much less frequently, *Atraphaxis*, *Persicaria*, *Polygonella*, *Avicularia*, *Pseudomollia* or *Duravia* (e.g. Nowicke and Skvarla 1977; Zhong-ze et al. 1999; Hong et al. 2005; Yasmin et al. 2010a, Yasmin et al. 2010b; Mosaferi and Keshavarzi 2011; Yurtseva et al. 2014; Paul and Chowdhury 2020; Kong et al. 2021; Raycheva et al. 2021; Başer et al. 2022), while studies on the genera *Fallopia* and *Reynoutria* are relatively sparse (Halbritter 1998; Yasmin et al. 2009; Heigl 2020, 2021; Paul and Chowdhury 2020). Moreover, no studies on the variability of pollen grains of these plants have been conducted to date.

The taxonomic utility of pollen characters of *F. convolvulus* and *F. dumetorum* from Pakistan was shown in the study of Yasmin et al. (2009). Brief descriptions of pollen grains of four *Reynoutria* and *Fallopia* species (*F. aubertii*, *F. baldschuanica*, *F. convolvulus*, *F. japonica* (*R. japonica*)) were provided by Halbritter (1998) and Heigl (2020, 2021). Paul and Chowdhury (2020) characterised pollen grains of *F. convolvulus* as part of a broader study on the pollen morphology of 32 taxa of subfamily Polygonoideae (Polygonaceae) from India.

Reynoutria japonica, *R. sachalinensis* and *R.* ×*bohemica* are considered transformer plant species in Europe (Chmura et al. 2015; Protopopova et al. 2015; Gentili et al. 2022). However, sexual reproduction is rarely observed in these taxa, which may be due to their pollen characteristics. Moreover, they grow in many different habitats (Bímová et al. 2004; Tiébré et al. 2008) that may play an important role in mediating the sexual recruitment of the plants. Consequently, the results of our research have an important practical aspect, as the reaction of the pollen of these invasive taxa to changing habitat conditions may affect the quality and effectiveness of their sexual reproduction.

Based on these premises, the primary objective of this study was to present a complete description of the pollen morphology of three *Reynoutria* taxa (*R. japonica* and *R. sachalinensis*, and their hybrids *R.* ×*bohemica*) – as was not found in the palynological literature – and investigate the range of intraspecific and interspecific variability of pollen grains of these invasive taxa, which has not been analysed to date. We also considered the influence of habitat conditions on the pollen morphology and variability and the share of male-fertile pollen-setting individuals in the Central European populations of the studied taxa. We tested the hypothesis that (1) there are individuals that set pollen in the studied populations of all *Reynoutria* taxa in Central Europe, and that (2) habitat conditions influence the morphological structure of knotweed pollen.

Material and methods

Plant material identification, collection and palynological analysis

The plant material (inflorescences) of three studied taxa: *Reynoutria japonica*, *R. sachalinensis* and *R.* ×*bohemica* was collected from August to October 2021, on dates of the optimal development phase of plants of individual taxa. The inflorescences were collected from 95 localities in the invasion range in Poland (*R. japonica* – 41 samples, *R. sachalinensis* – 30 samples and *R.* ×*bohemica* – 24 samples (Appendices 1, 2)), which corresponds to the range of occurrence of individual *Reynoutria* taxa in the study area (Zając and Zając 2001, 2019). The selection of localities was preceded by field reconnaissance conducted in previous years (some sites were recognized during earlier research), and data on the distribution of knotweeds collected from available sources, such as published research (e.g. Tokarska-Guzik et al. 2010; Bzdęga et al. 2012, 2022), herbarium data (e.g. Tokarska-Guzik et al. 2017) and unpublished data (mainly original author's data). Regarding population size, similar stands of *Reynoutria* taxa were selected for the study, although minor regional variations in the area occupied by each taxon favor *R. japonica* and *R.* ×*bohemica*. The determination of the knotweed was based on their morphological characteristics such as leaf shape and size, trichome type and morphology, flower number in the bunch, length of the stamens in relation to the perianth and the size of the panicle, which are well described as traits that are diagnostic for the identification of *Reynoutria* taxa (Beerling et al. 1994; Fojcik and Tokarska-Guzik 2000; Bailey et al. 2009; Tokarska-Guzik et al. 2017; Mereďa et al. 2019; Bzdęga et al. 2022). At each site, five ramets from several clumps were selected, and from each of them, three inflorescence clusters were collected. The collected plant material was stored at the Department of Botany and Forest Habitats at the Poznań University of Life Sciences.

In accordance with the study by Wrońska-Pilarek et al. (2015), each sample (n = 95) consisted of 30 randomly selected, mature and correctly formed pollen grains. The pollen grains were prepared for light microscopy (LM) and scanning electron microscopy (SEM) using the acetolysis method described by Erdtman (1952, 1960). The inflorescences were placed in tubes and then centrifuged with glacial acetic acid. Pollen grains were mixed with the acetolysis solution, which consisted of nine parts acetic anhydride and one part concentrated 95% sulphuric acid. The mixture was then heated to boiling and kept in a water bath for 2–3 min. Samples were centrifuged in the acetolysis mixture, washed with acetic acid and centrifuged again. The pollen grain samples were then mixed with 96% ethyl alcohol and centrifuged four times, with processed grains subsequently divided into two parts: one half of the samples was immersed in glycerin jelly for LM, while the other was placed in 96% ethyl alcohol in preparation for SEM. The pollen morphological observations were carried out using a scanning electron microscope (Zeiss Evo 40) and a digital light microscope (Levenhuk D870T) equipped with a camera and software enabling accurate measurements of the pollen grain.

We analysed the pollen grains for 11 quantitative characteristics: length of the polar axis (P) and equatorial diameter (E), length of the ectoaperture (Le), the distance between apices of two ectocolpi (d), thickness of the exine along the polar axis (Exp), thickness of the exine along the equatorial axis (Exe), as well as the ratios P/E, Le/P, Exp/P, Exp/E and d/E (apocolpium index P.A.I). The pollen shape classes (LA/SA ratio) were adopted based on the classification proposed by Erdtman (1952): suboblate (0.75–0.88), oblate-spheroidal (0.89–0.99), spheroidal (1.00), prolate-spheroidal (1.01–1.14), subprolate (1.15–1.33), prolate (1.34–2.00) and perprolate (> 2.01). The following qualitative features were also analysed: pollen outline and shape, exine ornamentation, operculum structure.

To estimate the amount of pollen in the tested plants, the pollen grain samples prepared for digital LM were used. Using a single-channel pipette, 0.5 ml of the glycerin jelly containing pollen grains from all pollen samples (n = 95) was transferred to a glass microscope slide, and a coverslip was applied. A digital LM (Levenhuk D870T) was used to count the number of pollen grains in all samples. As most samples contained more than 1000 pollen grains, the amount of pollen was subsampled (in all samples, all pollen grains were counted for ten areas of 1 mm^2, evenly distributed over the coverslip).

The palynological terminology used in the study follows Punt et al. (2007) and Halbritter et al. (2018).

Habitat analysis

The habitat type was defined at each site from which plant material was collected based on expert knowledge. Due to the great diversity of these areas, the habitats were grouped into seven categories corresponding to the plant communities of Matuszkiewicz (2022) (Table 1). The majority of samples were gathered from typical anthropogenic sites (60), mainly roadsides and railway embankments (42 samples), and other areas, including various types of urban and post-industrial wastelands, parks and gardens (18). The second group consisted of samples collected from semi-natural habitats, such as non-forested edges of large and small rivers and drainage ditches – hereinafter referred to as the edges of watercourses (22) – abandoned meadows (7) and forests (6) (in order of increasing habitat fertility: mesic mixed coniferous forest (1), mesic mixed broadleaved forest (2), mesic broadleaved forest (1) and floodplain forest (2)).

Table 1. Types of habitats from which the *Reynoutria* plant material originated, with corresponding classes of plant communities according to Matuszkiewicz (2022).

No.	Habitat type	Classes of plant communities corresponding to habitats	*R. japonica*	*R. sachalinensis*	*R. ×bohemica*
1	anthropogenic roadsides and other anthropogenic sites	*Stellarietea mediae* R.Tx., Lohm. et Prsg. 1950, *Artemisietea vulgaris* Lohm., Prsg. et R.Tx. in R.Tx. 1950	29	22	9
2	abandoned meadows and wastelands	*Molinio-Arrhenatheretea* R.Tx. 1937, *Artemisietea vulgaris* Lohm., Prsg. et R.Tx. in R.Tx. 1950, *Stellarietea mediae* R.Tx., Lohm. et Prsg. 1950	1	4	2
3	edge of the watercourses	*Phragmitetea australis* R.Tx. et Prsg. 1942, *Scheuchzerio-Caricetea nigrae* (Nordh. 1937) R.Tx. 1937, *Artemisietea vulgaris* Lohm., Prsg. et R.Tx. in R.Tx. 1950, *Stellarietea mediae* R.Tx., Lohm. et Prsg. 1950	9	2	11
4	mesic mixed coniferous forest	*Vaccinio-Piceetea* Br.-Bl. 1939, *Quercetea robori-petraeae* Br.-Bl. et R.Tx. 1943	1	0	0
5	mesic mixed broadleaved forest	*Querco-Fagetea* Br.-Bl. et Vlieg. 1937 (moderately fertile communities)	0	1	1
6	mesic broadleaved forest	*Querco-Fagetea* Br.-Bl. et Vlieg. 1937 (fertile communities)	0	1	0
7	floodplain forest	*Salicetea purpureae* Moor 1958, *Querco-Fagetea* Br.-Bl. et Vlieg. 1937	1	0	1
Total number of samples			41	30	24

Statistical analysis

One-way ANOVA with Tukey's post hoc test was used to compare the number of pollen grains in the studied samples (logarithm-transformed) between three *Reynoutria* taxa. Multivariate analysis of variance (MANOVA) was conducted to test for differences in three *Reynoutria* taxa as well as between habitat types for each *Reynoutria* taxon separately. In the MANOVA, an error structure including independent variables within the samples was added. Next, linear discriminant analysis (LDA) was used to analyse variation within and between *Reynoutria* taxa and habitat types. Box's M test was used to verify the multivariate normality and homogeneity of variance–covariance matrices. Next, mixed ANOVA's with Tukey's post hoc test were used to compare pollen traits among the three *Reynoutria* taxa and habitats. The Bonferroni correction method was used for multiple comparisons of habitats. The samples were included as a random effect on each model to control for the non-independence of the site where pollen grains were collected. Shapiro–Wilk's normality test was used to verify whether the residuals were normal for each pollen trait. The homogeneity of variance was tested using Bartlett's test; all the pollen traits were found to have a normal distribution. Although mixed ANOVA is generally robust to moderate imbalances, extreme disparities in sample sizes can lead to inflated Type I error rates or reduced statistical power, especially in pairwise comparisons. To mitigate this, we applied a Bonferroni correction for multiple comparisons, which helps control for the risk of false positives arising from the imbalanced samples. Moreover, the relationships between the 11 observed traits were estimated using Pearson's linear correlation coefficients based on the means for *R. japonica*, *R. sachalinensis* and *R. ×bohemica* separately. The significant relationship between traits ($p < 0.05$) was presented on heat maps. The analyses were carried out in R 4.0.2 (R Core Development Team 2020): the linear discriminant analysis was carried out using the MASS package (Venables and Ripley 2002), the mixed ANOVA using the lme4 (Bates et al. 2015) and car (Fox and Weisberg 2011) packages, and the data visualisation using the ggplot2 package (Wickham 2016).

Results

Number of pollen grains in the studied samples

All the tested specimens of knotweed (n = 95) developed male-fertile flowers containing pollen. Pollen grains were found in 41 specimens of *R. japonica*, 30 specimens of *R. sachalinensis* and 24 specimens of *R.* ×*bohemica*. The total number of pollen grains on a 10-mm^2 surface was 1–622 (5.79 ± 16.53) in *R. japonica*, 3–807 (217.54 ± 276.77) in *R.* ×*bohemica* and 1–1560 (236.57 ± 331) in *R. sachalinensis*.

The ANOVA indicated significant differences in the number of pollen between the three *Reynoutria* taxa ($F_{2,92} = 15.78$, $p < 0.0001$). Tukey post hoc tests indicated significant differences between *R. japonica* and both other taxa (Fig. 1). The least-numerous pollen samples were found in *R. japonica*, where 68.3% of samples had 5 or fewer pollen grains across a 10-mm^2 surface, versus 30% in *R. sachalinensis* and 12.5% in *R.* ×*bohemica*. At the same time, the lowest number (9.8% – 4 specimens) of samples containing over 100 pollen grains was found in *R. japonica*, versus 41.7% in *R.* ×*bohemica* and 50% in *R. sachalinensis* (Fig. 1).

General morphological description of pollen

In total, 2850 *Reynoutria* pollen grains were analysed (1230 of *R. japonica*, 900 of *R. sachalinensis* and 720 of *R.* ×*bohemica*). The studied pollen grains were tricolporate, isopolar monads (Fig. 2A–J, Table 2), mostly small (10–25 μm; 70%) and rarely medium sized (25.1–50 μm; 30%), with small pollen grains accounting for 81% of pollen in *R. sachalinensis*, 67% in *R japonica*, and 58% in *R.* ×*bohemica*.

The mean length of the polar axis (P) was 23.88 (11.90–44.15) μm. As the analysed pollen had a very small range of average values for trait P (from 23.26 to 24.52 μm), most of the *Reynoutria* pollen grains belonged to the upper limit of small pollen. The hybrid (*R.* ×*bohemica*) developed longer and wider pollen grains than the parental species *R. japonica* and *R. sachalinensis*. The values of trait P were 23.26 (14.96–44.15) μm in *R. sachalinensis*, 23.97 (16.09–40.33) μm in *R. japonica* and 24.52 (11.90–37.50) μm in *R.* ×*bohemica* (Table 2).

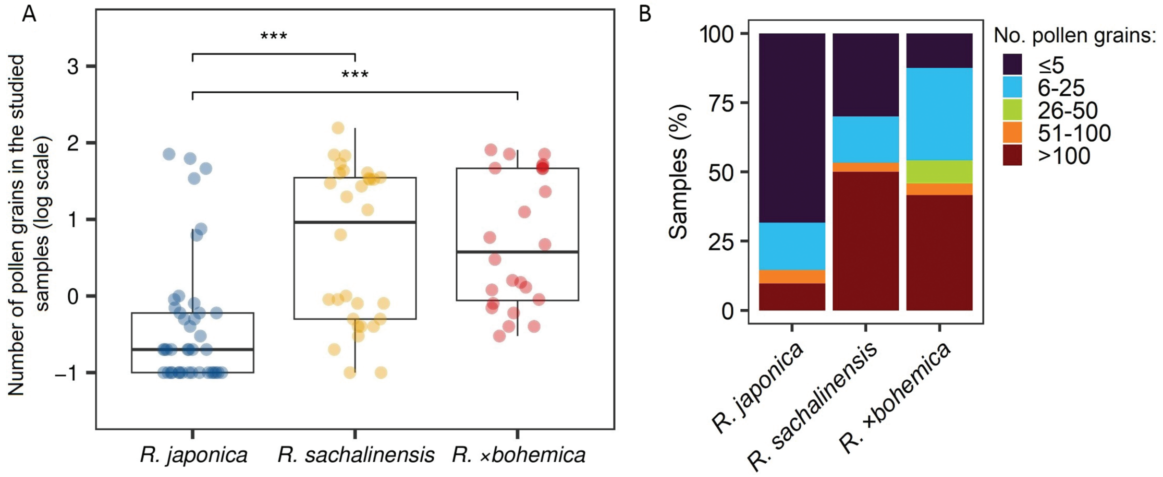

Figure 1. **A** boxplot of the number of pollen grains in the studied samples (log scale) of *Reynoutria* taxa **B** frequency of number of pollen-grain categories in the studied samples of *Reynoutria* taxa. Each point represents a single observation. Asterisks indicate significant differences (***P < 0.001).

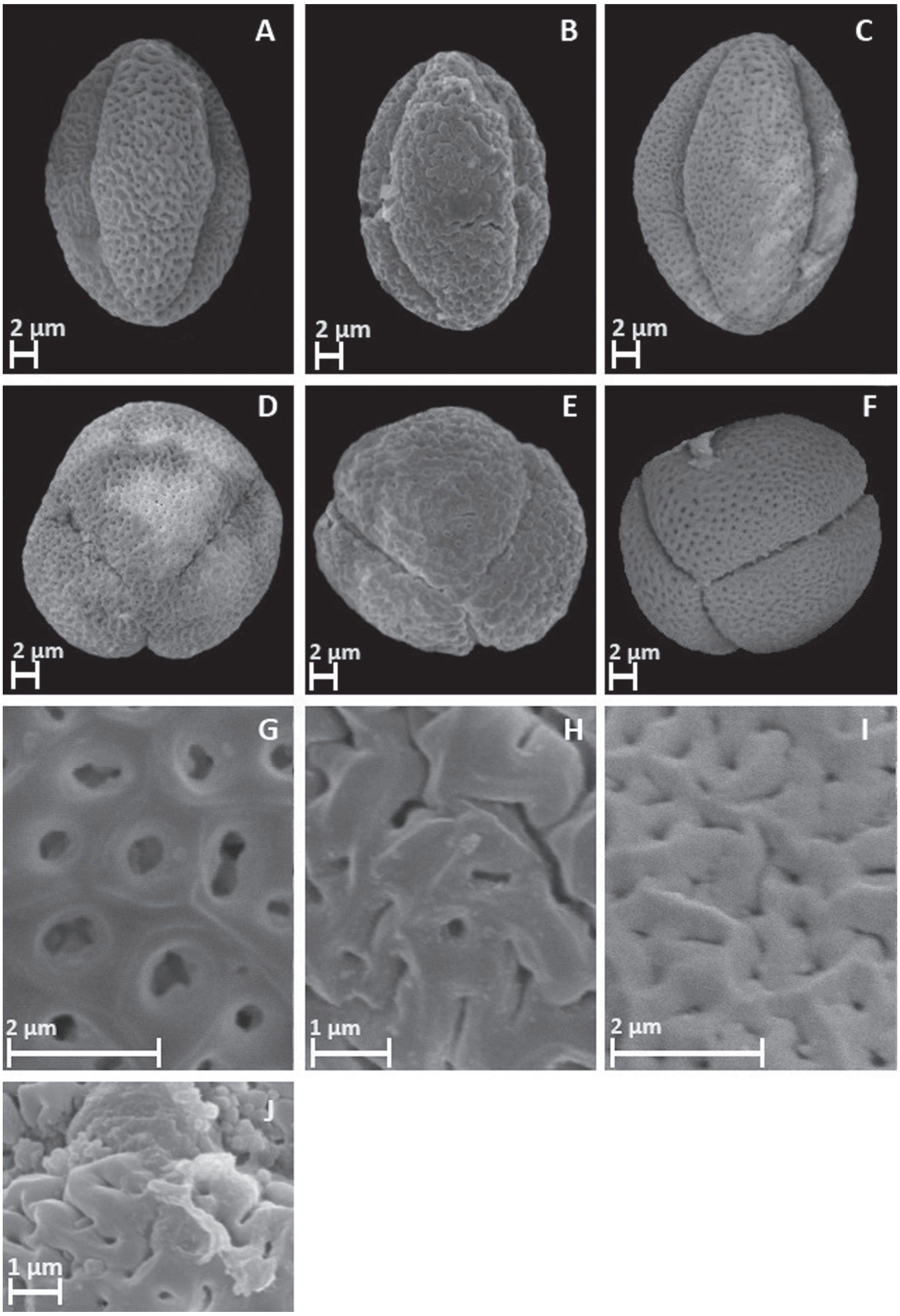

Figure 2. Pollen equatorial and polar views and exine ornamentation under scanning electron microscopy (SEM) **A–C** equatorial view of *R. japonica*, *R. sachalinensis*, *R.* ×*bohemica* **D–F** polar view and three colpori of *R. japonica*, *R. sachalinensis*, *R.* ×*bohemica* **G–I** exine ornamentation of *R. japonica*, *R. sachalinensis*, *R.* ×*bohemica* **J** operculum in *R. sachalinensis*.

The mean value of trait E was 22.91 (9.73–36.47) µm. The values of this trait were 23.62 (11.06–34.05) µm in *R.* ×*bohemica*, 22.87 (9.73–36.47) in *R. japonica* and 22.40 (10.97–34.32) µm in *R. sachalinensis*. The narrowest range of E variation was found in *R. japonica* (16.09–40.33 µm), and the widest in *R. sachalinensis* (14.96–44.15 µm) (Table 2). The outline in the polar view was mostly circular, and in the equatorial view mostly elliptic and rarely circular (Fig. 2A–F).

Table 2. The results of the mixed ANOVAs for pollen grain trait differences between studied *Reynoutria* taxa.

	R. japonica					***R. sachalinensis***					***R. ×bohemica***					**F**	**df**	**p**
	mean	**SD**	**min**	**max**	**CV (%)**	**mean**	**SD**	**min**	**max**	**CV (%)**	**mean**	**SD**	**min**	**max**	**CV (%)**			
P	23.969	3.125	16.090	40.330	13.04	23.264	2.603	14.960	44.150	11.19	24.518	3.005	11.900	37.500	12.26	7.14	2,2850	0.001
Le	19.447	3.033	7.350	32.720	15.60	19.224	2.330	10.180	39.150	12.12	19.582	2.774	7.900	31.650	14.17	0.68	2,2850	0.51
d	7.914	1.782	3.070	17.430	22.51	7.359	1.646	3.340	16.260	22.37	8.055	2.399	2.230	17.730	29.78	5.92	2,2850	0.004
E	22.866	3.068	9.730	36.470	13.42	22.399	2.386	10.970	34.320	10.65	23.624	3.015	11.060	34.050	12.76	7.08	2,2850	0.001
Exp	1.369	0.244	0.850	2.120	17.80	1.445	0.291	0.670	2.350	20.17	1.408	0.314	0.630	2.600	22.31	3.17	2,2850	0.046
Exe	1.482	0.235	0.940	2.180	15.86	1.607	0.294	0.820	2.500	18.30	1.460	0.356	0.480	2.400	24.37	8.34	2,2850	<0.001
P/E	1.053	0.095	0.691	2.382	9.05	1.042	0.094	0.764	1.881	9.00	1.044	0.112	0.711	1.745	10.72	1.57	2,2850	0.21
Le/P	0.811	0.060	0.341	1.000	7.42	0.827	0.052	0.429	1.000	6.29	0.799	0.061	0.381	1.000	7.58	11.5	2,2850	<0.001
d/E	0.347	0.069	0.163	0.887	20.01	0.329	0.066	0.153	0.601	20.19	0.340	0.090	0.128	0.699	26.42	2.93	2,2850	0.05
Exp/P	0.058	0.012	0.029	0.112	20.59	0.062	0.012	0.029	0.101	19.51	0.058	0.014	0.025	0.119	24.22	7.01	2,2850	0.002
Exp/E	0.061	0.013	0.033	0.161	21.64	0.065	0.013	0.030	0.112	20.04	0.060	0.015	0.026	0.128	25.29	5.38	2,2850	0.006

The P/E ratio (pollen shape) varied, averaging 1.05 (0.69–2.38). *Reynoutria* pollen grains were mostly prolate spherical (55.1% – 1570 pollen grains) or oblate spherical (30.8% – 879), rarely subprolate (12% – 342), and very rarely suboblate (1% – 28), prolate (0.9% – 27), oblate (0.1% – 3) or perprolate (0.04% – 1). Very similar results were obtained when analysing the distribution of pollen shape classes in individual taxa. In each of the taxa, prolate spherical pollen was found to be the most numerous (*R. japonica* – 59.9%, *R. sachalinensis* – 53.1%, *R. ×bohemica* – 43.1%), while oblate spheroidal pollen was the second most abundant shape, followed by subprolate pollen (Table 2).

The mean apocolpium index P.A.I (d/E ratio) was 0.34 and ranged from 0.13 (in *R. ×bohemica*) to 0.89 (in *R. japonica*). The lowest mean values of this index were recorded in *R. sachalinensis* (0.33), while the highest, which occurred in *R. japonica*, were similar (0.35) (Table 2).

The mean exine thickness was 1.46 (0.48–2.6) μm. The exine was thickest in *R. sachalinensis* (Exp – 1.45 μm and Exe – 1.61 μm) and thinnest in *R. japonica* (Exp – 1.37 μm) and *R. ×bohemica* (Exe – 1.46 μm). The mean exine thickness consisted of about 0.058 (0.02–0.12) of the P and 0.08 (0.02–0.14) of the E. The similarity of these results indicated a more or less equal exine thickness along the ends, thick regular with an irregular course and thick, slightly flattened walls of lumina, and lumina with small diameters in the pollen grains (Table 2).

Pollen grains usually possess three apertures, or 'colpori'. Colpi were arranged meridionally, regularly, were more or less evenly spaced, and were usually long (19.41 (7.35–39.15) μm) and very narrow, usually widening in the central (pori) area (Fig. 2A–F, Table 2). On average, the length of colpi constituted 81% of the polar axis length. Sometimes the colpi were so long that they met in the polar area, as in *R. ×bohemica* (Fig. 2A–F). The shortest colpi were found in *R. ×bohemica* (7.35 μm), while the longest was found in *R. sachalinensis* (39.15 μm). Sculpturing of ectocolpus membranes was close to microgranulate (Fig. 2J). The operculum observed in the studied taxa was minor, elliptical or spheroidal, slightly convex and situated above the porus, usually in the central part of the ectocolpus. Its sculpture was psilate and often corrugated (Fig. 2J).

Under SEM, the exine ornamentation of *R. sachalinensis* was rugulate, fossulate perforate, while that of *R. japonica* and *R. ×bohemica* was reticulate perforate (Fig. 2G–I). In *R. sachalinensis*, rugulae or fossulae were elongated, wide, flattened with irregular, geometric outlines and irregularly arranged. The perforations were small, quite sparse and irregularly distributed with different diameters (usually 0.1–0.2 μm). The reticulum of *R.* ×*bohemica* had wide muri – which were flattened at the top and had an irregular course – and lumina of various diameters and irregular outlines. The perforations were located at the bottom of the lumina and were very numerous, small and of similar diameter (usually 0.2 μm), as well as usually appearing circular or elliptical in outline. The reticulum of *R. japonica* consisted of wide, irregularly-shaped muri with an irregular, undulating course and walls narrowing at the top. The lumina had irregular outlines and varied diameters (usually 1.5 μm), usually larger than those in *R.* ×*bohemica*. Very numerous perforations were located at the bottom of the lumina, with quite large diameters (usually 0.20–0.40 μm, up to 0.80 μm), larger than those in *R.* ×*bohemica*.

The MANOVA revealed clear-cut, significant differences in terms of pollen grain traits between studied *Reynoutria* taxa (F = 7.67, Pillai = 1.01, $p < 0.001$). The mixed ANOVA also indicated significant differences in pollen traits between *Reynoutria* taxa, except for Le and P/E (Fig. 3, Table 2). Tukey's post hoc tests indicated significant differences in P, d, E, Exe, Le/P, Exp/P and Exp/E between *R.* ×*bohemica* and *R. sachalinensis*, and in P, d, Exe, Exp, d/E, Le/P, Exp/P and Exp/E between *R. japonica* and *R. sachalinensis* (excluding E). E significantly differed between *R.* ×*bohemica* and *R. japonica*.

The LDA showed that *R. sachalinensis* was more separate from *R.* ×*bohemica* and *R. japonica* (Fig. 4). The first two linear discriminant (LD) functions accounted for 100% of the total variability between the three *Reynoutria* taxa.

The most significant positive, linear relationships with the first LD were found for Exe (0.508) and Le/P (0.574), while a negative relationship was found for P (0.469) (Figs 5–7). The second LD was significantly positively correlated with E (0.242) and Exp (0.272), and negatively correlated with P/E (0.241). For *R. japonica*, strong significant positive correlations were found between Le and P (0.90), E and P (0.95),

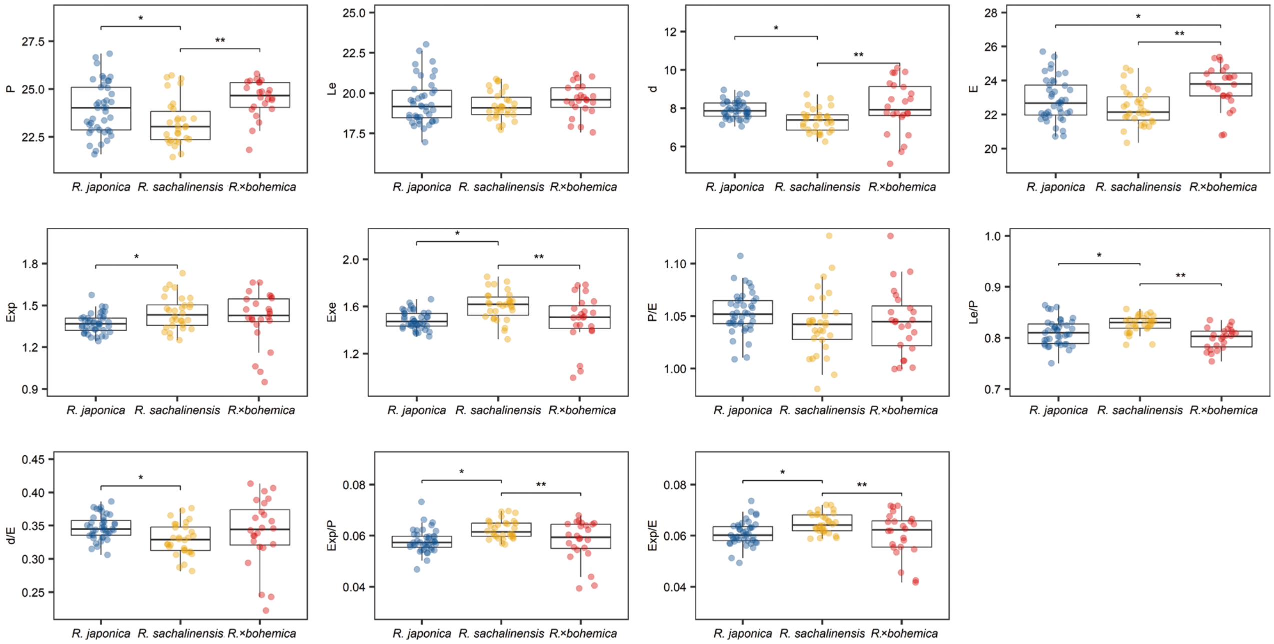

Figure 3. Boxplots of the 11 pollen grain traits analysed in the studied *Reynoutria* taxa. The points represent the mean values from each sample. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$).

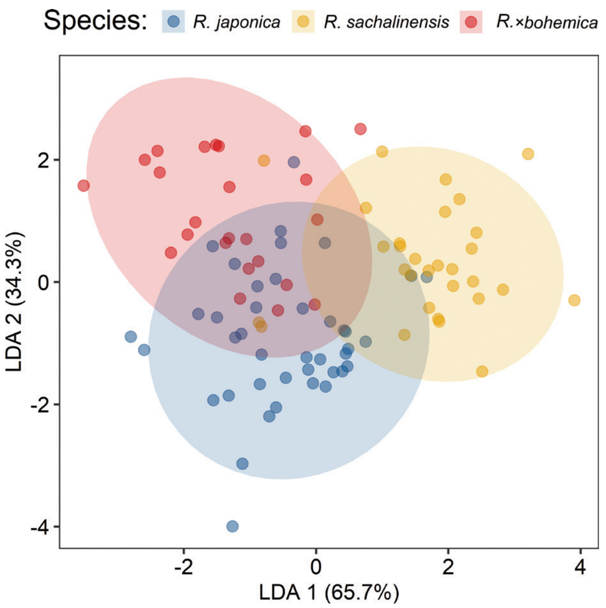

Figure 4. Biplots of linear discriminant function one (LD1) and two (LD2) for predicted variables of pollen grain traits of the studied *Reynoutria* taxa. The points represent values from each sample.

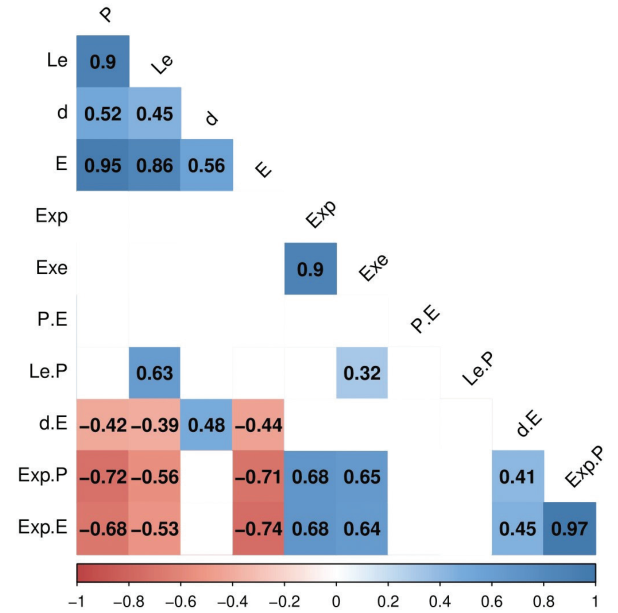

Figure 5. Heatmap for Pearson's correlation coefficients between observed traits for *R. japonica*. Significant coefficients are presented ($p < 0.05$).

Exp/E and Exp/P (0.97), as well as Exe and Exp (0.90), while strong negative correlations were observed between Exp/E and E (-0.74) and Exp/P and P (-0.72). For *R. sachalinensis*, strong significant positive correlations were found between Le and P (0.92), Exe and Exp (0.91), as well as Exp/E and Exp/P (0.89), while the most significant negative correlations were found between Le/P and P (-0.48) as well as between Le/P and P/E (-0.47). For *R.* ×*bohemica*, strong significant positive correlations were found between d/E and d (0.95), Exp and Exe (0.96), Exp/P and Exp (0.94), as well as Exp/E and Exp/P (0.98), while the most significant negative correlations were found between P/E and E (-0.73), Le/P and P/E (-0.60), as well as P/E and Le (-0.52). The other significant correlations are presented in Figs 5–7.

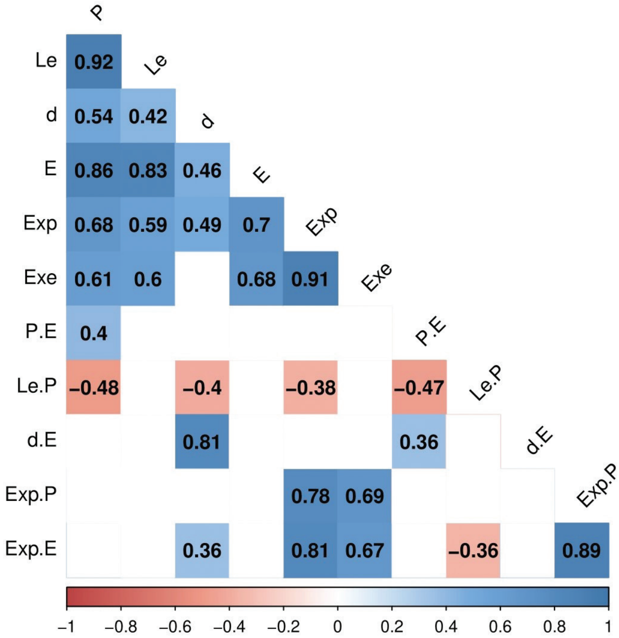

Figure 6. Heatmap for Pearson's correlation coefficients between observed traits for *R. sachalinensis*. Significant coefficients are presented (p < 0.05).

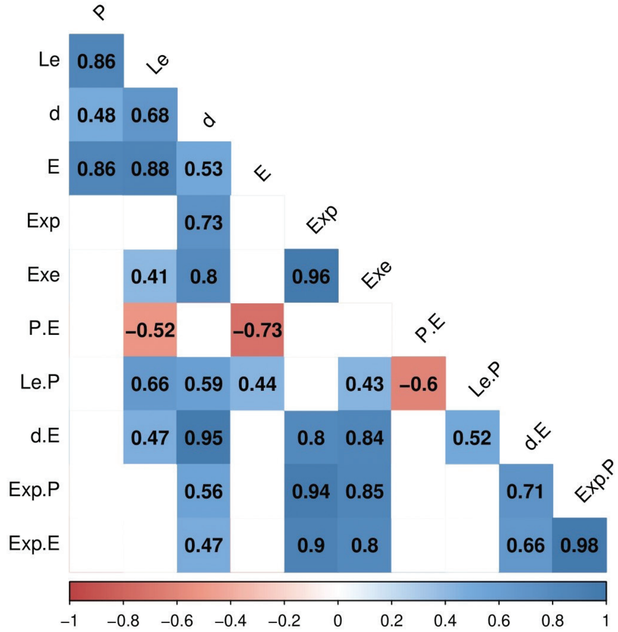

Figure 7. Heatmap for Pearson's correlation coefficients between observed traits for *R.* ×*bohemica*. Significant coefficients are presented (p < 0.05).

Pollen reaction to various habitat types

The MANOVA revealed significant differences in pollen grain traits of *Reynoutria* taxa between habitat types (F = 1.84, Pillai = 0.70, p = 0.011 and F = 6.79, Pillai = 0.06, p < 0.0001, respectively, for *R.* ×*bohemica* and *R. japonica*), except for *R. sachalinensis* (F = 1.26, Pillai = 0.03, p = 0.189).

The mixed ANOVA for *R. japonica* indicated significant differences in P, Le, E, Exe, Exp/P and Exp/E between habitats. The more conservative multiple comparison with Bonferroni correction showed significant differences between anthropogenic habitats and floodplain forest for P, Le, E, Exp/P and Exp/E, as well as between watercourse edges and floodplain forest for Exp/P and Exp/E (Fig. 8). For *R. sachalinensis*, no significant differences in pollen traits between habitats were indicated (Fig. 9), while the results for *R. ×bohemica* indicated significant differences in P, d, Exp, Exe, d/E, Exp/P and Exp/E between habitats. The more conservative multiple comparison with Bonferroni correction showed a significant difference between anthropogenic habitats and the edges of watercourses for d, Exp, Exe, d/E, Exp/P and Exp/E traits as well as between anthropogenic habitats and floodplain forest, and between meadows and floodplain forest, for P traits (Fig. 10).

The LDA for *R. japonica* showed that pollen samples collected in mixed coniferous forest, meadows and floodplain forest were separate from those of other habitats (Fig. 11A). In *R. sachalinensis*, on the other hand, pollen grains collected from each habitat were similar to each other (Fig. 11B). In *R. ×bohemica*, samples collected in anthropogenic and watercourse-edge habitats were slightly separate from each other; separations were also visible for single points between floodplain forest and meadow habitats (Fig. 11C).

The first two LDs accounted for 79.4%, 81.2% and 89.2% of the total variability between the individual habitats, respectively, for *R. japonica*, *R. sachalinensis* and *R. ×bohemica*. For *R. japonica*, the most significant positive, linear relationship with the first LD was found for Exe (0.526), while a negative relationship was found for d (0.435). The second LD was significantly positively correlated with Le (0.401), and negatively with Exp/E (0.424). For *R. sachalinensis*, the most significant positive linear relationship with the first LD was found for Le/P (0.403), while negative relationships were found for E (0.627) and Exp (0.574). The second LD was significantly positively correlated with P/E (0.376). For *R. ×bohemica*, the most significant negative linear relationships with the first LD were found for P (-0.639) and E (-0.484).

The average values of pollen traits in *R. japonica* were analysed (Table 3). Averages for the traits P, Le and E were highest in anthropogenic habitats (P – 24.28 µm, Le – 19.71 µm, E – 23.11 µm) and lowest in the mixed coniferous forest (P – 21.59 µm, Le – 16.96 µm, E – 20.71 µm). The mean values of trait d were higher at the edges of watercourses, at 7.96 µm, and lowest for the meadow habitat, at 7.14 µm. The average values of the traits Exp and Exe were higher in the floodplain forest (Exp – 1.49 µm, Exe – 1.66 µm) and lowest in the watercourse-edge habitat (Exp – 1.35 µm, Exe – 1.46 µm). The mean values for trait P/E were the same (1.05) for all studied habitats except meadows (1.04). The value of Le/P was higher in floodplain forest (0.82) and lowest in mixed coniferous forest (0.79). The mean value of the trait d/E was higher in mixed coniferous forest (0.38) and lowest in meadow habitat (0.33), while the ratios Exp/P and Exp/E were similar in all habitats.

The mean values of the traits P, Le, d, E, Exp and Exe for *R. sachalinensis* were higher in anthropogenic habitats (P – 23.46 µm, Le – 19.33 µm, d – 7.46 µm, E – 22.60 µm, Exp – 1.46 µm, Exe – 1.62 µm) and lower in mixed broadleaved forest (P – 22.19 µm, Le – 18.48 µm, d – 6.73 µm, E – 21.64 µm, Exp – 1.34 µm, Exe – 1.50 µm) (Table 3). The described values of trait P/E were higher at the edges of watercourses (1.06) and lower in broadleaved forest (1.01). The average value of the trait Le/P was slightly higher in broadleaved forest (0.84) and lower in anthropogenic habitats (0.82). The mean values of the traits d/E, Exp/P and Exp/E for *R. sachalinensis* were similar in all habitats.

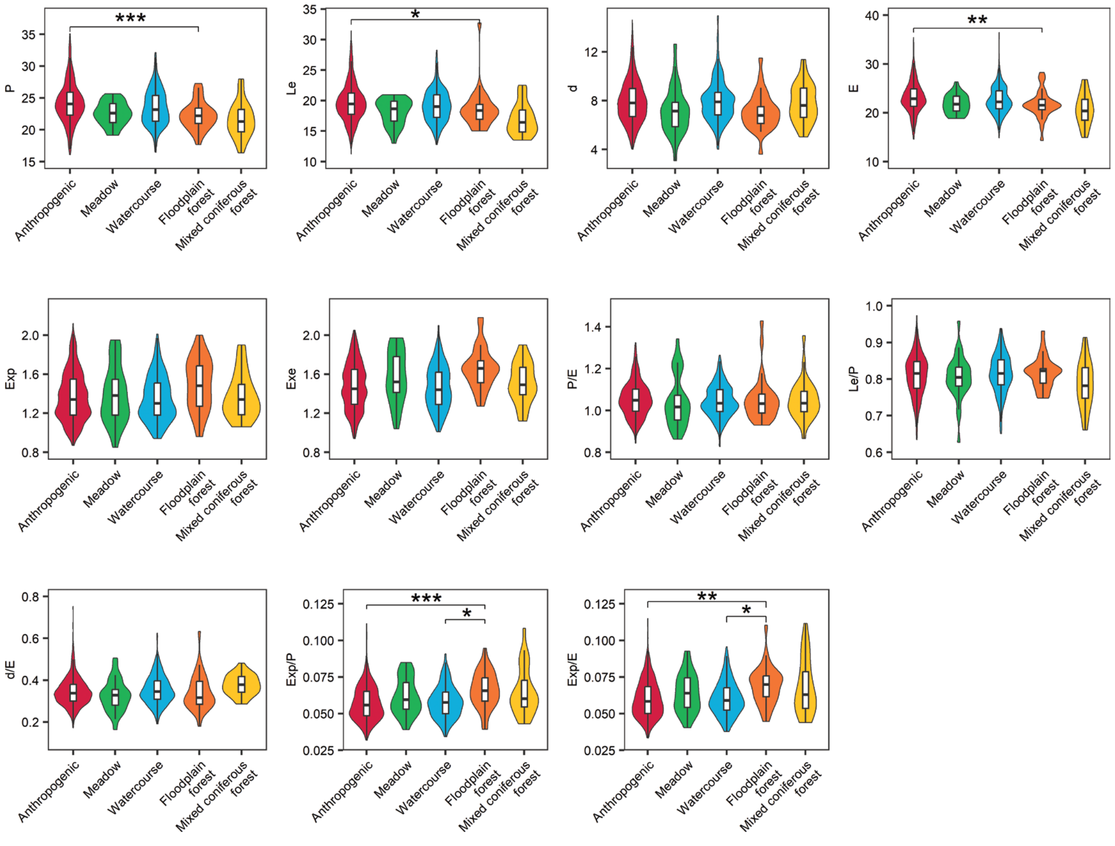

Figure 8. Boxplot and density plot of 11 pollen grain traits of *R. japonica* in relation to the habitat types studied. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, ***P < 0 .001).

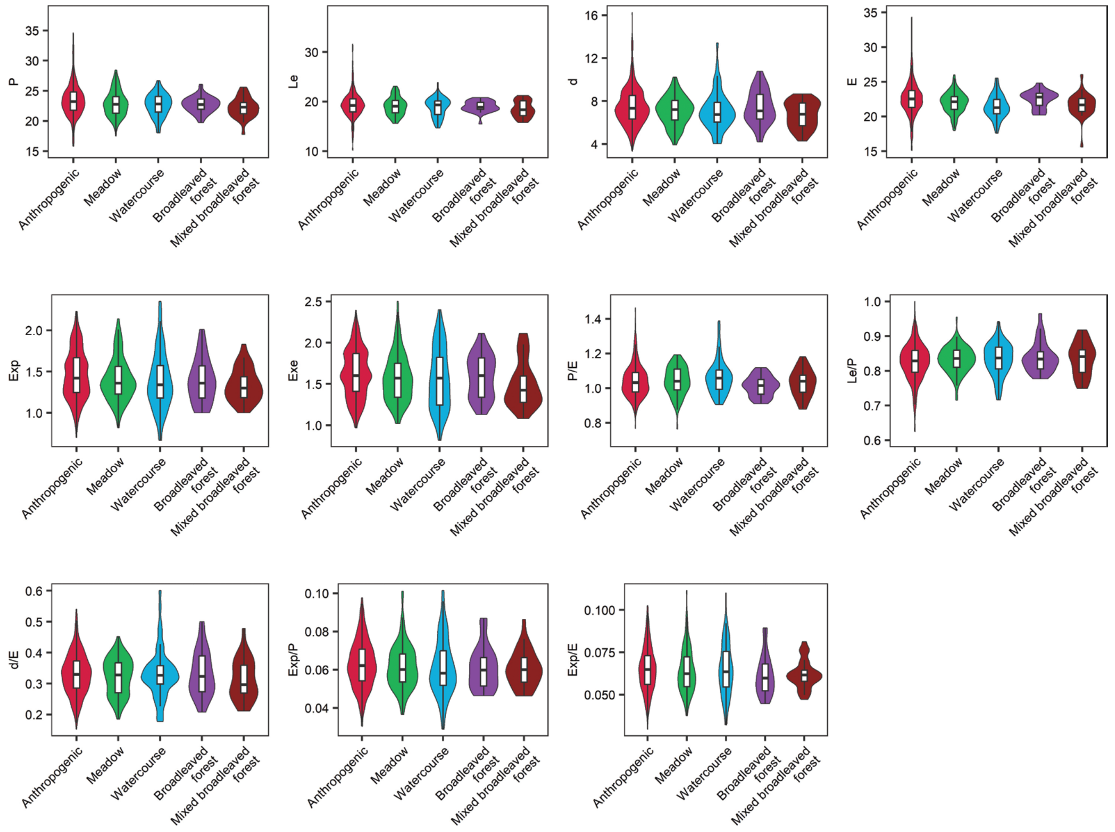

Figure 9. Boxplot and density plot of 11 pollen grain traits of *R. sachalinensis* in relation to the habitat types studied.

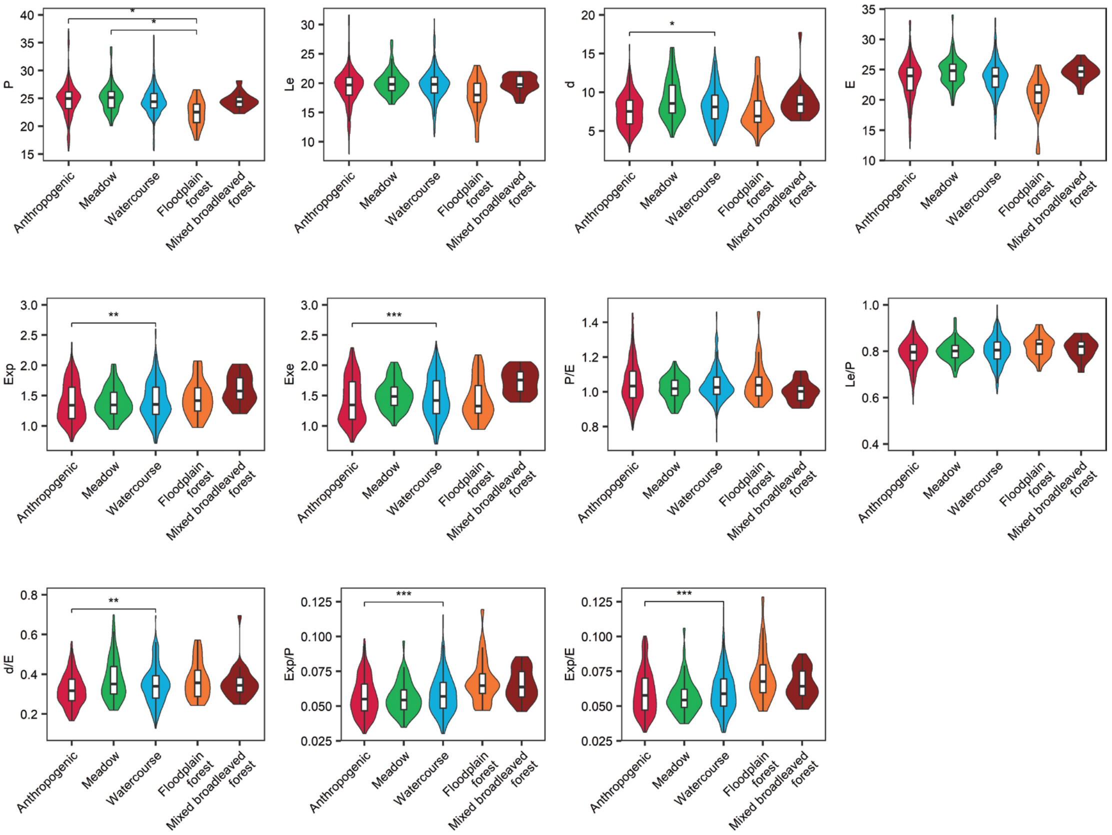

Figure 10. Boxplot and density plot of pollen grain traits of *R.* ×*bohemica* in relation to the habitat types studied. Asterisks indicate significant differences (*P < 0.05, **P < 0.01, ***P < 0.001).

The mean values of the traits P, Le, d and E in *R.* ×*bohemica* were higher in meadows (P – 25.09 μm, Le – 20.09 μm, d – 9.23 μm, E – 24.68 μm) and lower in floodplain forest (P – 21.82 μm, Le – 17.92 μm, E – 7.77 μm), except for trait d, which was lower in anthropogenic habitats (7.56 μm). The values of the traits Exp and Exe were higher in mixed broadleaved forest (Exp – 1.60 μm, Exe – 1.73 μm) and lower in anthropogenic habitats (Exp – 1.38 μm, Exe – 1.42 μm), while those of trait P/E were higher in anthropogenic habitats and floodplain forest (1.06), and lower in mixed broadleaved forest (1.00). The mean values of the ratios Le/P and d/E were slightly higher in floodplain forest (0.82 and 0.37, respectively) and lower in anthropogenic habitats (Le/P – 0.79, d/E – 0.32). The mean values of the traits Exp/P and Exp/E for *R.* ×*bohemica* were similar in all habitats (Table 3).

Discussion

Reproductive strategies influence the establishment, adaptive responses and invasive potential of alien species (Rejmánek and Richardson 1996; Grotkopp et al. 2002; Barrett et al. 2008; Barrett 2011; Gioria et al. 2023). Yet, surprisingly little is known about the sexual reproduction of *Reynoutria* taxa within their invasion range. Most researchers agree that *R. sachalinensis* and *R.* ×*bohemica* reproduce sexually very rarely (Pyšek et al. 2003; Tiébré et al. 2007; Bailey et al. 2009; Engler et al. 2011). For a long time, it was also pointed out that there is no generative reproduction in *R. japonica*, due to the lack of male-fertile plants in Europe (Bailey 1994; Beerling et al. 1994; Hollingsworth and Bailey 2000). The vast majority

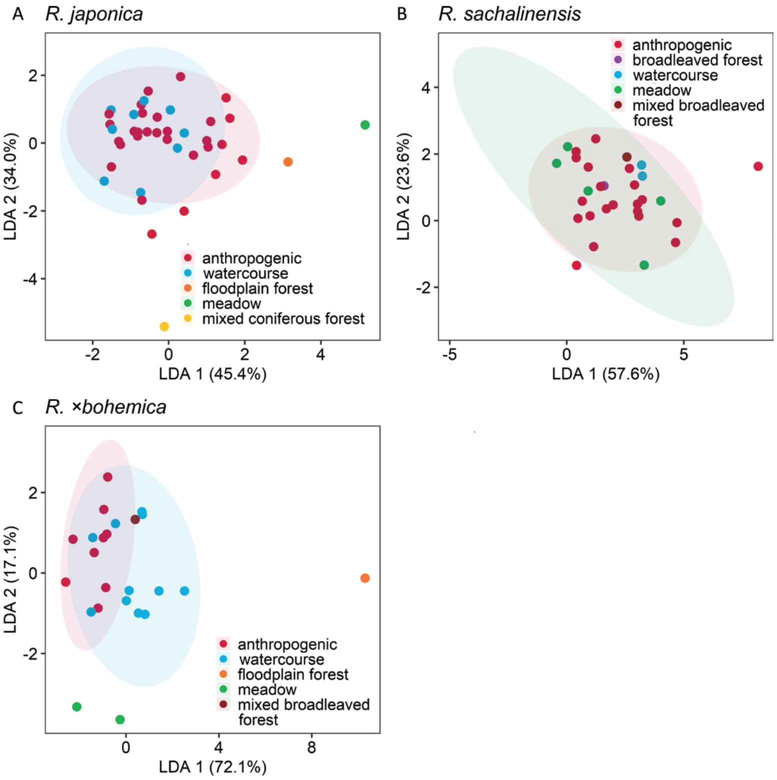

Figure 11. Biplots of linear discriminant function one (LD1) and two (LD2) for predicted variables of pollen grain traits in relation to habitat types separately for **A** *R. japonica* **B** *R. sachalinensis*, and **C** *R. ×bohemica*.

of genetic studies conducted on *R. japonica* individuals from England, France, Germany and the Czech Republic have confirmed this hypothesis and shown that the species is composed of one massive female clone in Western and Central Europe (Bailey and Stace 1992; Hollingsworth et al. 1998; Hollingsworth and Bailey 2000; Mandák et al. 2005; Bailey et al. 2009; Krebs et al. 2010).

Surprisingly, different results were obtained in genetic studies of knotweed populations in another part of Central Europe (in Poland). Studies conducted on *R. japonica* showed polymorphism between individuals of the species (Bzdęga et al. 2012). The researchers concluded that this result may be the effect of multiple genetic mutations, detected at greater levels than were possible before the development of a research technique (AFLP markers) with adequate sensitivity, or resulting from several episodes of *R. japonica* introduction in this area, which could have led to the broadening range of diversity observed in the studied populations. Attention was also drawn to the fact that the detected genetic diversity of the population of *R. japonica* may have resulted from the existence of sexual reproduction of this species in Central Europe (Bzdęga et al. 2012). Studies on the genetic variability of populations of three *Reynoutria* taxa from the native and Polish invasion ranges confirmed the advantage of clonal growth over sexual reproduction in the invasion range, except for populations where the analysed taxa cohabitated. The presence of such populations containing multiple genotypes and displaying a very low level of clonality raised the possibility of sexual reproduction potentially taking place at this stand (Bzdęga et al. 2016). Research on soil seed banks conducted in Poland confirmed the germination of *Reynoutria* seeds and the development of seedlings (and then mature plants) in soil monoliths taken from 'mixed' populations, i.e. those in which individuals with two types of flowers co-occurred, or in populations composed of two or three taxa (Koszela 2013; Tokarska-Guzik et al. 2017).

Table 3. The results of the ANOVAs for pollen grain trait differences between habitats, conducted separately for all studied *Reynoutria* taxa.

R. japonica	**F**	**df**	**p**	**Anthropogenic**					**Meadow**					**Watercourse**					**Floodplain forest**					**Mixed coniferous forest**				
Traits				**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**
P	4.66	4,1225	0.002	24.28	3.17	16.09	35.95	13.07	22.56	1.82	19.12	25.64	8.08	23.52	2.78	16.44	32.11	11.82	22.85	3.97	17.66	40.33	17.37	21.59	2.78	16.37	27.98	12.87
Le	4.50	4,1225	0.002	19.71	3.10	11.22	31.43	15.75	18.17	2.12	13.00	20.96	11.65	19.10	2.69	7.35	28.26	14.10	18.67	3.26	15.02	32.72	17.45	16.96	2.58	13.50	22.52	15.19
d	0.32	4,1225	0.864	7.95	1.79	4.01	17.43	22.56	7.14	1.92	3.07	12.62	26.97	7.96	1.66	4.02	14.94	20.88	7.45	2.36	3.60	17.03	31.67	7.81	1.56	5.02	11.37	19.94
E	4.03	4,1225	0.005	23.11	3.12	14.60	35.43	13.52	21.79	1.94	18.83	26.32	8.91	22.55	2.82	9.73	36.47	12.52	21.73	2.86	14.32	28.25	13.15	20.71	3.22	14.91	26.78	15.53
Exp	1.40	4,1225	0.241	1.37	0.25	0.87	2.12	17.90	1.39	0.29	0.85	1.95	20.94	1.35	0.23	0.94	2.01	16.95	1.49	0.27	0.96	2.00	17.87	1.37	0.23	1.06	1.90	16.92
Exe	3.61	4,1225	0.009	1.48	0.24	0.94	2.05	16.03	1.58	0.26	1.04	1.97	16.76	1.46	0.22	1.01	2.10	15.09	1.66	0.21	1.27	2.18	12.72	1.51	0.21	1.12	1.90	13.92
P/E	0.21	4,1225	0.934	1.05	0.09	0.69	1.77	8.34	1.04	0.12	0.86	1.34	11.80	1.05	0.11	0.71	2.38	10.70	1.05	0.11	0.93	1.43	10.18	1.05	0.09	0.87	1.36	8.98
Le/P	0.49	4,1225	0.739	0.81	0.06	0.60	1.00	7.31	0.80	0.06	0.63	0.96	7.57	0.81	0.06	0.34	0.94	7.86	0.82	0.04	0.75	0.93	5.17	0.79	0.06	0.66	0.91	8.18
d/E	1.63	4,1225	0.177	0.34	0.07	0.17	0.75	19.38	0.33	0.08	0.16	0.51	23.35	0.36	0.08	0.19	0.89	21.29	0.34	0.09	0.18	0.63	25.14	0.38	0.05	0.29	0.48	12.83
Exp/P	5.30	4,1225	0.001	0.06	0.01	0.03	0.11	20.69	0.06	0.01	0.04	0.08	20.05	0.06	0.01	0.03	0.09	18.75	0.07	0.01	0.04	0.09	18.89	0.07	0.02	0.04	0.11	24.87
Exp/E	4.66	4,1225	0.002	0.06	0.01	0.03	0.11	21.24	0.06	0.01	0.04	0.09	20.51	0.06	0.01	0.04	0.16	21.55	0.07	0.01	0.04	0.11	19.04	0.07	0.02	0.04	0.11	27.39
R. sachalinensis	**F**	**df**	**p**	**Anthropogenic**					**Meadow**					**Watercourse**					**Broadleaved forest**					**Mixed broadleaved forest**				
Traits				**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**
P	0.71	4,895	0.591	23.46	2.79	14.96	44.15	11.88	22.89	2.10	17.48	28.44	9.17	22.64	1.86	18.03	26.67	8.23	22.71	1.33	19.72	26.06	5.86	22.19	1.69	17.76	25.59	7.62
Le	0.57	4,895	0.684	19.33	2.51	10.18	39.15	12.97	19.04	1.78	15.58	23.09	9.35	18.87	1.88	14.67	23.84	9.96	19.03	1.12	15.48	20.78	5.91	18.48	1.60	15.77	21.19	8.64
d	0.79	4,895	0.545	7.46	1.69	3.34	16.26	22.70	7.09	1.34	3.92	10.23	18.94	7.08	1.78	4.02	13.41	25.10	7.44	1.57	4.20	10.76	21.13	6.73	1.22	4.29	8.66	18.11
E	1.16	4,895	0.354	22.60	2.59	10.97	34.32	11.48	21.98	1.50	17.98	25.98	6.84	21.41	1.65	17.56	25.52	7.71	22.48	1.24	20.20	24.80	5.54	21.64	1.71	15.59	26.04	7.92
Exp	0.73	4,895	0.583	1.46	0.29	0.70	2.23	20.08	1.40	0.26	0.82	2.14	18.24	1.39	0.35	0.67	2.35	25.18	1.39	0.27	1.00	2.01	19.32	1.34	0.20	1.00	1.83	15.11
Exe	0.51	4,895	0.731	1.62	0.29	0.97	2.39	17.76	1.57	0.29	1.02	2.50	18.36	1.56	0.37	0.82	2.40	23.60	1.59	0.26	1.13	2.11	16.24	1.50	0.28	1.08	2.11	18.81
P/E	0.74	4,895	0.576	1.04	0.10	0.77	1.88	9.34	1.04	0.08	0.76	1.19	7.77	1.06	0.10	0.90	1.39	9.26	1.01	0.06	0.91	1.12	5.59	1.03	0.08	0.88	1.18	7.46
Le/P	0.28	4,895	0.883	0.82	0.06	0.43	1.00	6.70	0.83	0.04	0.71	0.95	4.42	0.83	0.05	0.72	0.94	5.59	0.84	0.04	0.78	0.96	5.24	0.83	0.05	0.75	0.92	5.71
d/E	0.19	4,895	0.939	0.33	0.07	0.15	0.54	19.99	0.32	0.06	0.18	0.45	18.30	0.33	0.08	0.18	0.60	24.25	0.33	0.07	0.21	0.50	22.34	0.31	0.06	0.21	0.48	20.58
Exp/P	0.28	4,895	0.889	0.06	0.01	0.03	0.10	19.48	0.06	0.01	0.04	0.10	18.38	0.06	0.01	0.03	0.10	24.03	0.06	0.01	0.05	0.09	18.37	0.06	0.01	0.05	0.09	15.51
Exp/E	0.36	4,895	0.837	0.07	0.01	0.03	0.10	19.96	0.06	0.01	0.04	0.11	19.86	0.06	0.02	0.03	0.11	23.55	0.06	0.01	0.04	0.09	19.84	0.06	0.01	0.05	0.08	13.40
R. ×bohemica	**F**	**df**	**p**	**Anthropogenic**					**Meadow**					**Watercourse**					**Floodplain forest**					**Mixed broadleaved forest**				
Traits				**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**	**mean**	**SD**	**min**	**max**	**CV**
P	3.04	4, 715	0.042	24.64	3.33	13.56	37.50	13.53	25.09	2.53	20.05	34.28	10.09	24.55	2.76	13.42	36.35	11.25	21.82	3.29	11.90	26.53	15.10	24.56	1.43	22.24	28.17	5.83
Le	0.84	4, 715	0.516	19.46	3.05	7.90	31.65	15.69	20.09	2.09	16.39	27.35	10.42	19.72	2.66	10.87	30.98	13.48	17.92	3.09	9.92	23.02	17.23	19.86	1.43	16.55	21.99	7.22
d	2.87	4, 715	0.046	7.56	2.14	2.23	16.17	28.29	9.23	2.68	4.18	15.79	29.07	8.21	2.45	3.09	15.86	29.88	7.77	2.60	3.04	14.56	33.48	8.76	2.13	6.30	17.73	24.31
E	2.11	4, 715	0.115	23.53	3.25	11.94	33.15	13.82	24.68	2.33	19.08	34.05	9.43	23.68	2.84	13.52	33.53	12.01	20.78	3.24	11.06	25.76	15.61	24.59	1.50	20.89	27.44	6.10
Exp	5.06	4, 715	0.004	1.38	0.32	0.67	2.38	23.16	1.39	0.24	0.94	2.02	17.63	1.41	0.32	0.63	2.60	22.87	1.46	0.29	0.97	2.07	20.12	1.60	0.24	1.20	2.02	15.30
Exe	11.52	4, 715	<0.0001	1.42	0.37	0.60	2.29	26.44	1.51	0.24	1.00	2.05	16.17	1.46	0.36	0.48	2.40	24.51	1.43	0.34	0.94	2.17	23.43	1.73	0.20	1.39	2.06	11.80
P/E	1.14	4, 715	0.368	1.06	0.13	0.78	1.74	12.50	1.02	0.07	0.88	1.17	6.64	1.04	0.10	0.71	1.63	9.81	1.06	0.11	0.91	1.46	10.86	1.00	0.06	0.90	1.12	5.96
Le/P	1.38	4, 715	0.276	0.79	0.06	0.38	0.93	7.99	0.80	0.04	0.69	0.94	5.56	0.80	0.06	0.62	1.00	7.77	0.82	0.05	0.71	0.91	5.74	0.81	0.04	0.71	0.88	5.49
d/E	4.16	4, 715	0.011	0.32	0.08	0.17	0.56	24.75	0.37	0.10	0.22	0.70	27.97	0.35	0.09	0.13	0.70	26.76	0.37	0.09	0.24	0.57	24.72	0.36	0.08	0.25	0.70	23.13
Exp/P	5.25	4, 715	0.004	0.06	0.01	0.03	0.10	24.28	0.06	0.01	0.03	0.10	20.63	0.06	0.01	0.02	0.12	24.61	0.07	0.02	0.05	0.12	23.76	0.07	0.01	0.05	0.09	16.81
Exp/E	4.9	4, 715	0.005	0.06	0.02	0.03	0.10	26.09	0.06	0.01	0.04	0.11	21.98	0.06	0.02	0.03	0.12	25.07	0.07	0.02	0.05	0.13	25.34	0.07	0.01	0.05	0.09	16.95

In Europe, knotweed seedlings in the wild were recorded e.g. in Slovenia, Belgium, Germany and Poland (Tiébré et al. 2007; Engler et al. 2011; Funkenberg et al. 2012; Strgulc Krajšek and Dolenc Koce 2015; Tokarska-Guzik et al. 2017; Bzdęga et al. 2022), and in North America in the United States and Canada (Forman and Kesseli 2003; Groeneveld et al. 2014), but the mortality of seedlings tested in the field experiment was very high (Kadlecová et al. 2024). This, however, seems to be the result of unfavourable environmental conditions during the germination (e.g. water stress) rather than an inappropriate genetic or cytological make-up (Mereďa et al. 2023; Kadlecová et al. 2024). In our research, we found that all three analysed taxa of the genus *Reynoutria* – including the commonly considered male-sterile *R. japonica* – produce pollen, which positively validates our first hypothesis. Moreover, male-fertile individuals of *R. japonica*, as in the populations of this species studied in North America (Forman and Kesseli 2003), do not appear to be an anomaly, as pollen grains were detected in the flowers of all examined *R. japonica* specimens. However, only in about 10% of them was the amount of pollen large – comparable to its amount in the heavily pollen-producing specimens of *R. sachalinensis* and *R.* ×*bohemica*.

Single male-fertile plants have already been observed in invasion populations of *R. japonica* in the United States, where at least one male-fertile plant was found to occur within the pollination distance of each female plant (Forman and Kesseli 2003); the same phenomenon was observed in Germany (Child and Wade 2000). Still, as Alberternst and Böhmer (2011) emphasise, this phenomenon seemed to be very rare. Our results indicating the occurrence of male-fertile individuals of *R. japonica* in Central Europe, including single individuals providing a large supply of pollen, are in line with these observations, but also clearly correspond to the results of genetic studies of the *R. japonica* in this area (Bzdęga et al. 2012, 2016). This also seems to indicate that the source of *R. japonica* polymorphism in Central Europe may be isolated cases of sexual reproduction in this species. Although the production of pollen itself does not determine, for example, its viability and germination (Dafni and Firmage 2000) or the possibility of effective pollen transfer and pollination (Waytes et al. 2022), the presence of fertile males certainly promotes effective sexual reproduction in the case of this species. Therefore, in such situations, as noted by Grimsby et al. (2007), more attention should be paid to the survival of *R. japonica* seeds and seedlings. Although the recruitment of knotweed seedlings in Europe is strongly dependent on climatic conditions (Beerling et al. 1994; Bailey et al. 2009; Kadlecová et al. 2024), which cause most seeds to rot in the ground (Bailey et al. 2009; Koszela 2013), it is known that an increase in the frequency of sexual reproduction is responsible for the increased ability of plants to adapt to environmental stochasticity (Lei 2010). This in turn can significantly increase invasiveness, which should be taken into account during the monitoring of *R. japonica* and other invasive species. As noted by Soll (2004), if extensive sexual reproduction in *R. japonica* is confirmed in natural conditions, it would be necessary to change the planning strategy for population control of this species at the landscape level.

Knowledge of the biology of the species, including its method of reproduction and spread, is crucial in developing effective methods to limit the phenomenon, given the increasing negative impact of invasive alien species on biodiversity, the economy and human quality of life (IPBES 2023). Taxa of the *Reynoutria* genus are considered as posing a significant environmental threat and are particularly difficult to eliminate, mainly due to their high ability to regenerate from underground rhizomes (even small fragments) and above-ground shoots (e.g. Aguilera et al. 2010; Alberternst and Böhmer 2011; Tokarska-Guzik et al. 2017; Švec et al. 2024). An increase in the share of sexual reproduction in the secondary range of these taxa may affect the effects of remedial actions taken. So far, dozens of control methods have been described for knotweed, some of which are still being tested (Dusz et al. 2021; Bzdęga et al. 2022, 2024, Švec et al. 2024). The descriptions of control methods quite rarely indicated the need to take into account sexual reproduction, pointing primarily to the possibility of seedlings appearing in 'mixed' populations, i.e. composed of two or three taxa. Our results regarding the possibility of sexual reproduction in *R. japonica* populations suggest the need to take this aspect into account when planning control methods for all knotweed populations. For example, the proposed long-term strategy for combating knotweed (Švec et al. 2024) should take into account actions that prevent knotweed from blooming/seed setting, controlling the possibility of seedlings appearing in gaps in dense knotweed canopies as a result of plant elimination measures, or planning the treatment of soil (in the event of its possible movement) which may potentially, in addition to the rhizomes, also contain viable knotweed seeds.

There are only a few publications regarding the pollen morphology of the genus *Fallopia* and *Reynoutria*. In the palynological studies carried out to date on species from the family Polygonaceae, representatives of this genus were usually not included (e.g. Nowicke and Skvarla 1977; Zhong-ze et al. 1999; Hong et al. 2005; Mosaferi and Keshavarzi 2011; Raycheva et al. 2021). According to Nowicke and Skvarla (1977) and Faegri et al. (2000), Polygonaceae species are very diverse in terms of their pollen features, especially in exine structure and ornamentation. The research of the above-cited authors and our results also confirm this assumption concerning the *Fallopia* and *Reynoutria* genus. The most important pollen features of this genus are the exine ornamentation type, polar-axis length (P) and pollen shape (P/E ratio). According to Hong et al. (2005), some species of *Fallopia* (e.g. *F. convolvulus*, *F. dumetorum*) show a dimorphic exine (smooth in the mesocolpium area, but punctate in the region of the colpi), as was earlier observed by Nowicke and Skvarla (1977). However, the described dimorphism did not occur in the taxa we examined.

To date, the pollen morphology of five *Fallopia* and *Reynoutria* taxa has been examined: *F. convolvulus* (Yasmin et al. 2009; Halbritter et al. 2018; Heigl 2020; Paul and Chowdhury 2020), *F. dumetorum* (Yasmin et al. 2009) and *F. aubertii*, *F. baldschuanica* and *F. japonica* (*R. japonica*) (Halbritter et al. 2018; Heigl 2020, 2021). The results of the present study and of the few studies cited above agree that the pollen grains of the genus are isopolar, tricolporate monads. According to palynologists, the pollen sizes are small (P = 21.00 μm in *F. convolvulus* and *F. dumetorum*; Yasmin et al. 2009) or, also in *F. convolvulus* (Paul and Chowdhury 2020), small or medium-sized (Halbritter 1998; Heigl 2020, 2021). Our research found both sizes of pollen, with a clear advantage of small pollen (70%) over medium-sized pollen (30%); additionally, the mean value of P was higher than those of the cited researchers, at 23.88 μm. These differences are probably due to the fact that we studied species other than those of the above-cited authors, but also to the very large sample of pollen grains measured in our study. The pollen shape (P/E ratio) described in other *Fallopia*/*Reynoutria* species was subprolate, sometimes prolate spheroidal (Yasmin et al. 2009), prolate spheroidal (Paul and Chowdhury 2020) or usually spheroidal according to Halbritter (1998) and Heigl (2020, 2021). Our results showed seven pollen shape types (mostly prolate spherical and oblate spherical, rarely subprolate, suboblate, prolate, oblate or perprolate). In the study of

Yasmin et al. (2009), the mean length of the ectocolpus (Le) was 12.0–12.8 μm; a similar result (12.11 μm) was obtained by Paul and Chowdhury (2020), while Heigl (2020) did not provide values for this feature. In our study, the average value of this feature was 19.41 μm. The exine thickness ranged from 2.00 μm in *F. dumetorum* to 3.8 μm in *F. convolvulus* (Yasmin et al. 2009), while our study found it to be 1.46 (0.48–2.6) μm in *Reynoutria* taxa. Based on SEM observations of exine ornamentation, Halbritter (1998) described that of *F. convolvulus* as psilate, microechinate and perforate, and that of *F. baldschuanica* as perforate, microechinate and microgemmate. On the other hand, Yasmin et al. (2009) distinguished two pollen types: an *F. convolvulus* type (microechinate-punctate) and *F. dumetorum* type (reticulate). Paul and Chowdhury (2020) reported the same exine type in *F. convolvulus* (microechinate-punctate). Here, we found that the exine ornamentation under SEM appeared rugulate and fossulate perforate in *R. sachalinensis* and reticulate perforate in *R. japonica* and *R. ×bohemica*. These are different types to those described above because the exine of these taxa has not been examined to date. This result confirms the opinion of Nowicke and Skvarla (1977) and Faegri et al. (2000) about the large diversity of exine ornamentation types in the genus *Fallopia* and *Reynoutria*.

All studied *Reynoutria* taxa are now widely naturalised in Europe and pose a threat to native ecosystems (Tokarska-Guzik et al. 2006; Gerber et al. 2008; Moravcová et al. 2011; Chmura et al. 2015), but understanding the conditions of their invasion in different habitats and the causes of these differences requires taking into account many factors (Bímová et al. 2004). Generally, research has shown that in Europe, three knotweed taxa invade similar habitats, although certain habitat preferences have been observed for each of them (Bímová et al. 2004; Tiébré et al. 2008; Tokarska-Guzik et al. 2017). Habitat patch colonisation dynamics and distribution patterns analysed at landscape scale showed that *R. japonica* seems to have a wide ecological range (Mandák et al. 2004; Tiébré et al. 2008; Aguilera et al. 2010) – according to Bímová et al. (2004), the widest among the three knotweed taxa. The species invades both anthropogenic and natural sites. It grows on roadsides, railway embankments, urban and post-industrial wastelands, parks, cemeteries and home gardens, but also invades natural sites (river banks, forest edges and thickets and forests, especially riparian forests) (Bímová et al. 2004; Mandák et al. 2004; Tokarska-Guzik et al. 2017). *R. sachalinensis* is rarer than *R. japonica* in the invasion range. The species is most often recorded in sites more or less influenced by human activity, like urban or disturbed areas (gardens, parks, railsides and roadsides), but sometimes also colonises riverbanks (Shaw 2008; Tokarska-Guzik et al. 2009, 2017; Chmura et al. 2015). The habitat requirements of *R. ×bohemica* are similar to those of the parental species, but Bímová et al. (2004) and Tiébré et al. (2008) showed particularly similar patterns of habitat selection between *R. japonica* and *R. ×bohemica*, and Bímová et al. (2004) indicated that in the Czech Republic, *R. ×bohemica* mainly occupies riverside habitats.

Our research found that, although pollen grains are considered the most conservative plant organs (Stace 1989), habitat conditions influence some pollen quantitative characteristics of *R. japonica* and *R. ×bohemica*. For *R. sachalinensis*, no significant differences in pollen traits between habitats were indicated; thus, our second hypothesis that habitat conditions affect the morphological structure of all knotweed pollen should be partially rejected. For *R. japonica*, differences in pollen characteristics between habitats were found in P, Le, E, Exe, Exp/P and Exp/E, and for *R.* ×*bohemica* in P, d, Exp, Exe, d/E, Exp/P and Exp/E. This indicates that P, Exp, E, Exp/P and Exp/E can be considered the knotweed pollen traits most strongly responsive to habitat conditions, which partially coincides with rarely obtained research results for other plant species. Wrońska-Pilarek et al. (2023) showed that in *Convallaria majalis*, Ex/LA and Ex are the pollen traits that respond most strongly to habitat conditions. It is therefore possible that exine thickness is a pollen trait that is sensitive to habitat properties, which is particularly important because the success of pollen-tube penetration depends on exine thickness in the colpus membrane (Wang and Dobritsa 2018); this means that the properties of the habitat may affect the fertilisation process.

These results also indicate that habitat conditions similarly affect the quantitative features of the morphological structure of *R. japonica* and *R.* ×*bohemica* pollen. These observations correspond to the results of the cluster analysis of genetic polymorphism between pairs of taxa, which showed that *R.* ×*bohemica* is much more similar to *R. japonica* than to *R. sachalinensis* (Bzdęga et al. 2012, 2016), a fact that may translate into the similar reaction of these taxa to diverse environmental conditions.

We obtained similar results by examining inter- and intraspecific variability of knotweed pollen growing in different habitats. We found that the pollen of *R. sachalinensis* collected from different habitats were similar, whereas there was some variability of pollen from *R. japonica* and *R.* ×*bohemica* growing in different conditions. In *R.* ×*bohemica*, differences were found between pollen samples collected from anthropogenic habitats and the edges of watercourses. Since, as noted by Bímová et al. (2004), the phenotypic variability of *R.* ×*bohemica* is greater than the variability of both parental taxa, perhaps this variability also translates into other, previously undescribed morphological features of this plant and affects the stronger reaction of this taxon's pollen to habitat conditions. In the case of both *R. japonica* and *R.* ×*bohemica*, individual plants growing in forests (including riparian forests, among other types) and in abandoned meadows produced different pollen from the other examined individuals. In Central Europe, the habitats most invaded by *Reynoutria* taxa are more often flooded than the other habitat types (Bímová et al. 2004), and in Belgium, Tiébré et al. (2008) demonstrated the importance of aquatic and humid habitats and linear landscape structures in the distribution patterns of *R. japonica* and *R.*×*bohemica*, which show high genetic similarity in such conditions (Bzdęga et al. 2012). It can therefore be assumed that individuals growing in wet habitats, such as the periodically flooded edges of watercourses, riparian forests or, for example, abandoned wet meadows, produce slightly different pollen to those growing in anthropogenic habitats. A detailed analysis of the average values of pollen traits showed that values of the P, Le and E for *R. japonica* and *R. sachalinensis* were highest in anthropogenic habitats, and for *R.* ×*bohemica* in abandoned meadows. We also found that for all examined taxa, the average values of these features were lowest in the forests. This means that *R. japonica* and *R. sachalinensis* develop the largest pollen in anthropogenic habitats, and *R.* ×*bohemica* in abandoned meadows, while the smallest pollen grains are formed by knotweeds growing in forests. This may be related to the fact that pollen grains under more intense desiccation stress during flowering periods tend to be larger (Ejsmond et al. 2011). It is therefore possible that in open areas (like anthropogenic habitats or meadows), where the substrate dries out faster, the desiccation stress is greater than in more stable forest communities, affecting the size of knotweed grains.

In light of the very complex habitat–plant relationships and, to our knowledge, the lack of a study linking pollen morphology with natural environmental factors, we were aware that the interpretation of the obtained results regarding the effect of habitat on pollen would be difficult. Another limitation of our study is the imbalance in the number of samples collected from each habitat type. Sample sizes were determined based on the natural distribution and abundance of *Reynoutria* taxa in the study area, and this imbalance reflects the actual occurrence of taxa in different habitats. Although this sampling design is ecologically relevant, it may introduce some statistical limitations, especially when comparing pollen traits across habitat types. Furthermore, the diversity of forest habitats in terms of environmental variables such as fertility and moisture may contribute to the variability of pollen traits. These factors may influence the results of the analyses, especially in habitats where environmental conditions are significantly different. This introduces another source of variability that should be considered when interpreting the results. We acknowledge this as a limitation and suggest that future studies could include a more controlled sampling of habitat variables to better isolate the effects of environmental factors on pollen traits. However, because the gametophyte stage is a key stage in the plant life cycle, it seems important to try to understand how environmental fluctuations affect its fitness (Matamoro-Vidal et al. 2016). Our studies indicate that certain morphological pollen traits of knotweeds are influenced by habitat conditions. Since we often assume that morphological traits are closely related to the functions they perform and vice versa (e.g. Lauder 1981), it seems that the focus of further research should be to determine whether changes in the morphological structure of pollen induced by habitat conditions affect its viability. This is especially important, because our study clearly indicates that the gametophytic phase of the plant life cycle of *Reynoutria* species may play an increasingly important role in the invasion process of these species, and that this may be partly determined by habitat conditions.

Conclusions

- The most important novel result of our research is demonstrating that in the studied knotweed populations in Central Europe, *R. japonica*, *R. sachalinensis* and *R.* ×*bohemica* produce pollen, although it was hitherto thought that in the invasion range, plants identified as *R. japonica* are male sterile.
- A clear difference was found in the amount of pollen among the examined taxa. The amount of *R. japonica* pollen was lower than that of the other two studied taxa, but approximately 10% of individuals of this species had abundant pollen. Further investigations are needed to determine what role, if any, sexual reproduction plays in the spreading system and evolution of *R. japonica* in Central Europe.
- Based on pollen morphological features, it was possible to distinguish *R. sachalinensis*, which has different exine ornamentation (rugulate, fossulate perforate), from the other two taxa (reticulate perforate).
- The following pollen traits showed the most pronounced response to different habitat conditions in the seven studied habitats: P, Exp, E, Exp/P and Exp/E. Of the three taxa, the hybrid *R.* ×*bohemica* showed the strongest response to habitat conditions.

- The greatest interspecific variability was found between *R. japonica* and *R. sachalinensis*, and the smallest between *R. japonica* and *R. ×bohemica*. The greatest intraspecific variability was demonstrated by *R. ×bohemica*, and the smallest by *R. sachalinensis*.
- We found that the reaction of pollen grains collected from seven different habitat types varied. Pollen grains of *R. sachalinensis* were more or less similar. In *R. japonica*, pollen samples collected in mixed coniferous forest, meadows and floodplain forest were separate from those of other habitats, and in *R. ×bohemica*, samples from anthropogenic and watercourse-edge habitats were slightly separate from each other; separations were also visible for single points between floodplain forest and meadow habitats. The reaction was not always clearly visible, probably because pollen grains, as the most conservative plant organs, respond slowly to changing habitat conditions.
- In our opinion, the finding of male-fertile *R. japonica* individuals in Central Europe has important implications for the management of knotweed in Europe and highlights the importance of regional research in understanding the biology of invasive plant species.

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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Conceptualization B.W and D.W.-P.; Methodology B.W., D.W.-P. and Ł.D; Software Ł.D., B.W. and D.W.-P.; Validation B.W. and D.W.-P.; Formal analysis Ł.D., B.W., D.W.-P.; Investigation B.W., D.W.-P., K.B, B.T.-G. and Ł.D.; Data curation B.W., K.B., B.T.-G, Z.D., A.G.; Writing—original draft preparation B.W., D.W.-P., K.B., Ł.D., Writing—review and editing B.W, D.W.-P., K.B., B.T.-G, Z.D.; Visualization Ł.D., B.W.

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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. List of research sites of *R. japonica*, *R. sachalinensis* and *R. ×bohemica* located in Poland.

Sample No.	Species	Localities	Geographical coordinates	Collector
1.1	*R. japonica*	Poland, Kaniów	49.954841°N, 19.055690°E	B. Tokarska-Guzik
1.2	*R. japonica*	Poland, Koźlice	51.105560°N, 14.983808°E	B. Tokarska-Guzik
1.3	*R. japonica*	Poland, Trzemienko	50.242639°N, 19.783250°E	B. Tokarska-Guzik
1.4	*R. japonica*	Poland, Poznań	52.409491°N, 16.984006°E	B. Wiatrowska
1.5	*R. japonica*	Poland, Pobiedziska	52.481637°N, 17.275364°E	B. Wiatrowska
1.6	*R. japonica*	Poland, Pobiedziska	52.480329°N, 17.284132°E	B. Wiatrowska
1.7	*R. japonica*	Poland, Gorzkie Pole	52.480755°N, 17.210946°E	B. Wiatrowska
1.8	*R. japonica*	Poland, Rakoniewice	52.136650°N, 16.271461°E	B. Wiatrowska
1.9	*R. japonica*	Poland, Lubięcin	51.886196°N, 15.872431°E	B. Wiatrowska
1.10	*R. japonica*	Poland, Głuszyca Górna	50.661948°N, 16.367022°E	P. Kiciński
1.11	*R. japonica*	Poland, Kowary	50.798055°N, 15.843444°E	Z. Dajdok
1.12	*R. japonica*	Poland, Wrocław Biskupin	51.096777°N, 17.110611°E	Z. Dajdok
1.13	*R. japonica*	Poland, Bolesławiec	51.267138°N, 15.554166°E	Z. Dajdok
1.14	*R. japonica*	Poland, Ząbkowice Śląskie	50.589527°N, 16.806916°E	Z. Dajdok
1.15	*R. japonica*	Poland, Oława	50.608083°N, 17.307500°E	Z. Dajdok
1.16	*R. japonica*	Poland, Wrocław-Żernik	51.124361°N, 16.915055°E	Z. Dajdok
1.17	*R. japonica*	Poland, Potasznia	51.539527°N, 17.493805°E	Z. Dajdok
1.18	*R. japonica*	Poland, Sławoszowice	51.533000°N, 17.307638°E	Z. Dajdok
1.19	*R. japonica*	Poland, Żmigródek	51.486388°N, 16.917222°E	Z. Dajdok
1.20	*R. japonica*	Poland, Trzebnica	51.319916°N, 17.059944°E	Z. Dajdok

Sample No.	Species	Localities	Geographical coordinates	Collector
1.21	*R. japonica*	Poland, Namysłów	51.080666°N, 17.710527°E	Z. Dajdok
1.22	*R. japonica*	Poland, Markowe	50.989750°N, 17.972666°E	Z. Dajdok
1.23	*R. japonica*	Poland, Brzeg	50.846361°N, 17.458361°E	Z. Dajdok
1.24	*R. japonica*	Poland, Wołczyn	51.010944°N, 18.068000°E	Z. Dajdok
1.25	*R. japonica*	Poland, Wiry	50.843611°N, 16.636972°E	Z. Dajdok
1.26	*R. japonica*	Poland, Wrocław-Strachocin	51.104166°N, 17.146944°E	Z. Dajdok
1.27	*R. japonica*	Poland, Solniki Wielkie	51.162808°N, 17.475097°E	Z. Dajdok
1.28	*R. japonica*	Poland, Ligota	49.885748°N, 18.947847°E	K. Bzdęga
1.29	*R. japonica*	Poland, Dzbanów	50.509698°N, 16.786601°E	K. Bzdęga
1.30	*R. japonica*	Poland, Dąbrowa Górnicza	50.316544°N, 19.229872°E	K. Bzdęga
1.31	*R. japonica*	Poland, Sucha Beskidzka	49.748925°N, 19.605571°E	K. Bzdęga
1.32	*R. japonica*	Poland, Zator	49.961216°N, 19.440823°E	K. Bzdęga
1.33	*R. japonica*	Poland, Szczekociny	50.637709°N, 19.755217°E	K. Bzdęga
1.34	*R. japonica*	Poland, Stare Czarnowo	53.324489°N, 14.684350°E	A. Gobber
1.35	*R. japonica*	Poland, Szczecin	53.366469°N, 14.729677°E	A. Gobber
1.36	*R. japonica*	Poland, Szczecin	53.389201°N, 14.514944°E	A. Gobber
1.37	*R. japonica*	Poland, Szczecin	53.408924°N, 14.558056°E	A. Gobber
1.38	*R. japonica*	Poland, Szczecin	53.361887°N, 14.737131°E	A. Gobber
1.39	*R. japonica*	Poland, Wrzosowo	54.013266°N, 14.804340°E	A. Gobber
1.40	*R. japonica*	Poland, Wolin	53.851410°N, 14.616641°E	A. Gobber
1.41	*R. japonica*	Poland, Skoszewo	53.781858°N, 14.631426°E	A. Gobber
2.1	*R. ×bohemica*	Poland, Poznań	52.407935°N, 16.956244°E	B. Wiatrowska
2.2	*R. ×bohemica*	Poland, Poznań	52.420542°N, 16.895445°E	B. Wiatrowska
2.3	*R. ×bohemica*	Poland, Poznań	52.433327°N, 16.911499°E	B. Wiatrowska
2.4	*R. ×bohemica*	Poland, Poznań	52.387607°N, 16.979838°E	B. Wiatrowska
2.5	*R. ×bohemica*	Poland, Poznań	52.418498°N, 16.907190°E	B. Wiatrowska
2.6	*R. ×bohemica*	Poland, Poznań	52.404153°N, 17.034627°E	B. Wiatrowska
2.7	*R. ×bohemica*	Poland, Poznań	52.420007°N, 16.959805°E	B. Wiatrowska
2.8	*R. ×bohemica*	Poland, Promno	52.475986°N, 17.209989°E	B. Wiatrowska
2.9	*R. ×bohemica*	Poland, Biskupice	52.460256°N, 17.179204°E	B. Wiatrowska
2.10	*R. ×bohemica*	Poland, Polanica Zdrój	50.417016°N, 16.511467°E	B. Tokarska-Guzik
2.11	*R. ×bohemica*	Poland, Skoczów	49.813271°N, 18.792147°E	B. Tokarska-Guzik
2.12	*R. ×bohemica*	Poland, Wilkanów	50.233900°N, 16.726300°E	B. Tokarska-Guzik
2.13	*R. ×bohemica*	Poland, Wilkowisko	49.754918°N, 20.259190°E	B. Tokarska-Guzik
2.14	*R. ×bohemica*	Poland, Radomierzyce	51.062083°N, 14.967150°E	B. Tokarska-Guzik
2.15	*R. ×bohemica*	Poland, Piskórka	51.988614°N, 21.020531°E	B. Tokarska-Guzik
2.16	*R. ×bohemica*	Poland, Izabelin	52.285350°N, 20.835167°E	B. Tokarska-Guzik
2.17	*R. ×bohemica*	Poland, Janowice Wielkie	50.880944°N, 15.925055°E	Z. Dajdok
2.18	*R. ×bohemica*	Poland, Bardo Śląskie	50.509444°N, 16.734000°E	Z. Dajdok
2.19	*R. ×bohemica*	Poland, Mościsko	50.782333°N, 16.588305°E	Z. Dajdok
2.20	*R. ×bohemica*	Poland, Wrocław-Biskupin	51.767222°N, 17.077777°E	Z. Dajdok
2.21	*R. ×bohemica*	Poland, Wrocław-Jarnołtów	51.123388°N, 16.841805°E	Z. Dajdok
2.22	*R. ×bohemica*	Poland, Międzyrzecze Górne	49.850545°N, 18.926569°E	K. Bzdęga
2.23	*R. ×bohemica*	Poland, Dąbrowa Górnicza	50.351958°N, 19.383935°E	K. Bzdęga
2.24	*R. ×bohemica*	Poland, Katowice	50.231923°N, 19.027429°E	K. Bzdęga
3.1	*R. sachalinensis*	Poland, Poznań	52.440737°N, 16.901670°E	B. Wiatrowska
3.2	*R. sachalinensis*	Poland, Poznań	52.439741°N, 16.910695°E	B. Wiatrowska
3.3	*R. sachalinensis*	Poland, Wierzonka	52.486721°N, 17.091999°E	B. Wiatrowska
3.4	*R. sachalinensis*	Poland, Istebna	49.603850°N, 18.902867°E	B. Tokarska-Guzik
3.5	*R. sachalinensis*	Poland, Smoleń	50.428804°N, 19.666947°E	B. Tokarska-Guzik
3.6	*R. sachalinensis*	Poland, Duszniki Zdrój	50.404733°N, 16.398750°E	B. Tokarska-Guzik

Sample No.	Species	Localities	Geographical coordinates	Collector
3.7	*R. sachalinensis*	Poland, Miedzianka	50.875833°N, 15.945100°E	B. Tokarska-Guzik
3.8	*R. sachalinensis*	Poland, Olszany	49.747900°N, 22.634933°E	B. Tokarska-Guzik
3.9	*R. sachalinensis*	Poland, Nowy Żytnik	54.252116°N, 16.686833°E	B. Tokarska-Guzik
3.10	*R. sachalinensis*	Poland, Boguszów-Gorce	50.751972°N, 16.182055°E	Z. Dajdok
3.11	*R. sachalinensis*	Poland, Karpacz	50.771305°N, 15.759416°E	Z. Dajdok
3.12	*R. sachalinensis*	Poland, Wrocław-Strachocin	51.103083°N, 17.143583°E	Z. Dajdok
3.13	*R. sachalinensis*	Poland, Łagiewniki	50.793527°N, 16.994555°E	Z. Dajdok
3.14	*R. sachalinensis*	Poland, Grodziszcze	50.797305°N, 16.565166°E	Z. Dajdok
3.15	*R. sachalinensis*	Poland, Wrocław-Żerniki	51.122333°N, 16.935555°E	Z. Dajdok
3.16	*R. sachalinensis*	Poland, Starościn	50.986194°N, 17.838944°E	Z. Dajdok
3.17	*R. sachalinensis*	Poland, Dąbrowa	50.971361°N, 17.796166°E	Z. Dajdok
3.18	*R. sachalinensis*	Poland, Międzyrzecze Górne	49.850363°N, 18.926083°E	K. Bzdęga
3.19	*R. sachalinensis*	Poland, Sucha Beskidzka	49.748925°N, 19.605571°E	K. Bzdęga
3.20	*R. sachalinensis*	Poland, Katowice	50.231102°N, 19.026277°E	K. Bzdęga
3.21	*R. sachalinensis*	Poland, Stare Brynki	53.301346°N, 14.560283°E	A. Gobber
3.22	*R. sachalinensis*	Poland, Żabnica	53.295000°N, 14.501470°E	A. Gobber
3.23	*R. sachalinensis*	Poland, Miłachowo	53.939224°N, 14.775219°E	A. Gobber
3.24	*R. sachalinensis*	Poland, Police	53.539068°N, 14.577298°E	A. Gobber
3.25	*R. sachalinensis*	Poland, Szczecin	53.452032°N, 14.531967°E	A. Gobber
3.26	*R. sachalinensis*	Poland, Nowe Warpno	53.723634°N, 14.282981°E	A. Gobber
3.27	*R. sachalinensis*	Poland, Mierzeszyn	54.199942°N, 18.418546°E	A. Gobber
3.28	*R. sachalinensis*	Poland, Egiertowo	54.237831°N, 18.200722°E	A. Gobber
3.29	*R. sachalinensis*	Poland, Mały Klincz	54.131388°N, 18.049467°E	A. Gobber
3.30	*R. sachalinensis*	Poland, Piaszno	54.098524°N, 17.359810°E	A. Gobber

Appendix 2

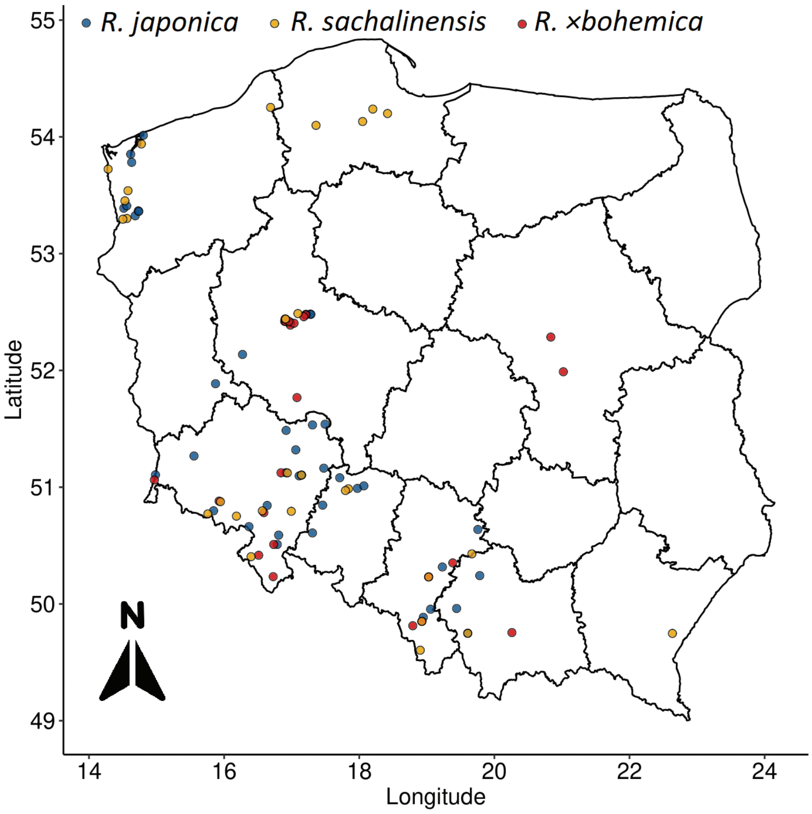

Figure A1. Localities of the *R. japonica*, *R. sachalinensis* and *R. ×bohemica* sites in the study area.

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Research Article

The fellowship of the fig continues its journey: Fig wasps (Hymenoptera, Chalcidoidea) associated with *Ficus microcarpa* in Greece

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Abstract

The number of alien species continues to climb uninterrupted with a proportion of them becoming invasive, impacting native biodiversity and socioeconomic parameters. Many alien species are plants, transported outside their native range, sometimes alongside their associated insects hitching a ride to new destinations. *Ficus microcarpa* L. (Moraceae) is a common ornamental plant in the Mediterranean, which has been found to host a large ecological network of associated chalcid wasps (also called fig wasps). Amongst them, the plant's pollinator *Eupristina verticillata* Waterston (Agaonidae), enhances the plant's successful pollination and subsequent germination, thus allowing it to establish viable populations and even become invasive in some parts of the world. Other associated wasps, also called non-pollinating fig wasps, have likewise followed and these are parasitoids, inquilines or gallers. These species can be either beneficial or injurious to *F. microcarpa*, with some even proposed as potential biological control agents mitigating the plant's spread. Seven fig wasp species have been reported from Greece, hitherto. Here, we present the first national survey of fig wasp fauna for Greece. We found 13 species, with six representing new records for the country (*Eufroggattisca okinavensis* Ishii, *Micranisa degastris* Chen, *Philotrypesis okinavensis* Ishii, *Philotrypesis taiwanensis* Chen, *Odontofroggatia quinifuniculus* Feng & Huang, *Sycophila curta* Chen) and two (*O. quinifuniculus*, *S. curta*) being reported outside their native range for the first time. *Philotrypesis emeryi* is removed from previous checklists upon molecular characterization of specimens as being *Philotrypesis okinavensis*. We discuss the distribution, introduction period and ecology of these fig wasps. Further, we present records of seedlings for *F. microcarpa* and discuss the potential implications of the fig wasp fauna for the establishment and control of this alien plant species.

Key words: Alien species, biological invasions, chalcid wasps, distribution, first records, ecological networks

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Introduction

International trade, along with globalisation and the constant transportation of people and goods around the world have greatly contributed to a rising number of species managing to reach areas beyond their natural distribution, a trend that continues uninterrupted (Hulme 2009; Seebens et al. 2017; Seebens 2019). A considerable number of these "alien" species have managed to establish viable populations and even become invasive in their newly-invaded areas (Wang 2014). Alien species can also trigger a series of adverse impacts on native biodiversity and ecosystem functions, human health and societal welfare, as well as negatively affecting global economy (Vilà and Hulme 2016; Mazza and Tricarico 2018; Haubrock et al. 2021). Biodiversity loss and pressures on native species by invasive alien species are evident *inter alia* through their competition with native and/or endemic species, alteration of trophic webs and transmissions of pathogens (Seebens et al. 2017; Chinchio et al. 2020). From a socioeconomic perspective, invasive alien species and their impacts have been thoroughly discussed in both the EU and the US (Pimentel et al. 2005; Kettunen et al. 2009; Haubrock et al. 2021).

Plant species have often been deliberately introduced in areas beyond their natural distribution for forestry, agricultural, medicinal or ornamental purposes, while there are also plenty of cases of unintentional introduction (Newsome and Noble 1986; Williamson 1996; Arianoutsou et al. 2010). The Mediterranean climate is suitable for the establishment of numerous alien plant species from tropical and sub-tropical as well as temperate areas (Bella 2014a) and their use as ornamentals in parks and gardens in Europe is increasing (Lopez-Vaamonde et al. 2010; Bella 2014b). Following on from introduction of their hosts, numerous insect species associated with introduced ornamentals have established in introduced areas (Bonnamour et al. 2023) where they are most commonly found in gardens and parks as well as other anthropogenic habitats (Lopez-Vaamonde et al. 2010). This broad trend is evident in Greece, as plant species such as *Eucalyptus* spp. have been associated with the unintentional introduction and establishment of various phytophagous chalcid wasps (including, but not limited to *Leptocybe invasa* Fischer & La Salle, 2004, *Ophelimus maskelli* Ashmead, 1900 and *Quadrastichodella nova* Girault, 1922) (Georgevits 1981; Kavallieratos et al. 2004; Anagnou-Veroniki et al. 2008).

The genus *Ficus* L. (Rosales, Moraceae) includes more than 800 species and is of remarkable ecological value, providing food or nesting sites to many vertebrates (Berg 1989; Shanahan et al. 2001; Wang 2014). The majority of *Ficus* species are distributed in Indomalaya, while around 150 species originate from each of the Afrotropics and the Neotropics (van Noort and Rasplus 2022). Fig tree fruits, known as "figs", are in fact an enclosed inflorescence, with numerous small flowers and are also termed "syconia" (Cook and Rasplus 2003). Figs can be viewed as a microhabitat for insect species that can be considered either as beneficial or injurious to the plant, depending on their trophic regime (Grandi 1961; Galil and Eisikowitch 1968b). Pollination of each *Ficus* species is carried out by species in the family Agaonidae (Hymenoptera, Chalcidoidea) and, in turn, these wasps depend on their host tree since they dwell and feed inside the figs (Galil and Eisikowitch 1968b; Cook and Rasplus 2003). This positive association known as a mutualism, is exploited by a series of non-pollinating species (Non-Pollinating Fig Wasps; NPFWs) of various trophic regimes (gallers, inquilines and parasitoids) (Chen et al. 1999; Cook and Rasplus 2003). Each *Ficus* species can host up to 30 species of fig wasps or more, providing us with micro-community ecological models of great interest (Compton et al. 1994; West et al. 1996; Cook and Rasplus 2003).

Known by various common names (Chinese/Malayan Banyan or Cuban/Indian Laurel), *Ficus microcarpa* L. is a monecious fig tree species, originating from sub-tropical and tropical areas of south-eastern Asia (Chen et al. 1999; Wagner et al. 1999; Bhandari and Cheng 2016). The same species can also be found in literature under the synonyms *F. nitida* Thunberg and *F. retusa* L. (Mifsud et al. 2012). Mature individuals can have 4–5 crops per year (Chen et al. 1999), but less in seasonal climates. This species has been introduced as an ornamental in various tropical and subtropical areas of the world and, in the Mediterranean, it is common throughout parks, private gardens and road sideways (Wang et al. 2015b; Demetriou et al. 2023). In introduced areas, it can occasionally be the cause of damage to infrastructure, especially old buildings and even expand into natural habitats as an invasive species (Ramírez and Montero 1988; McKey 1989; Beardsley 1998; Tsintides et al. 2002; Mifsud 2014; Wang et al. 2015b; Demetriou et al. 2023).

Phytophagous insect species associated with *F. microcarpa* can be divided into two main subcategories, depending on the parts of the tree they exploit. Along with some other insects, a paraphyletic group of wasps of the superfamily Chalcidoidea are called fig wasps and are associated with the figs. Elsewhere on the tree, insects feed on the leaves, twigs and branches. They belong to various insect orders (Coleoptera, Hemiptera, Hymenoptera, Lepidoptera, Thysanoptera) (van Noort and Rasplus 2022) and include some fig wasps that have apparently switched from galling figs to galling leaves and stems. Amongst these, described from outside its putative native range, *Josephiella microcarpae* Beardsley & Rasplus, 2001, has managed to spread throughout the Mediterranean and has been recently discovered from Greece (Crete, Rhodes) (Beardsley and Rasplus 2001; Kalaentzis et al. 2023; EK unpublished data). Other species, such as *Gynaikothrips ficorum* (Marchal, 1908) (Thysanoptera, Phlaeothripidae) along with its predator *Montandoniolla moraguesi* (Puton, 1896) (Hemiptera, Anthocoridae) and *Macrohomotoma gladiata* Kuwayama, 1908 have also managed to follow their host plant as pests and expand their distribution throughout southern Greece (Antonatos et al. 2015; Malumphy and Guillem 2020; EK, unpublished data). In turn, some of these fig tree pests have also been followed by their parasitoids, such as *Psyllaephagus macrohomotoma* Singh & Agarwal, 1993 and *Psyllaephagus schauffi* Japoshvili, 2023 (both parasitising *M. gladiata* in Greece and/or Cyprus) (Japoshvili et al. 2023).

Fig wasps can be further divided into trophic categories: those whose larvae feed on plant tissue inside galled ovules (phytophagous species) and those that fulfil their larval development at the expense of the phytophagous species (parasitoids, inquilines, secondary gallers and even hyper-parasitoids) (Compton et al. 2009; Segar and Cook 2012; Chen et al. 2013; Wang et al. 2015a). The most prominent phytophagous species is one of the pollinators of *F. microcarpa*, *Eupristina verticillata* Waterston 1921, which assists its host plant's reproduction by allowing it to produce fertile seeds in ripe figs, which, in turn, are carried away with the help of birds or even ants (Kaufmann et al. 1991). As a result, *F. microcarpa* has managed to escape urban habitats and, alongside its pollinator, it has a presence in all biogeographic regions, except for Antarctica (Wang et al. 2015a). Other fig wasps that inhabit the figs belong to families Epichrysomallidae, Eurytomidae, Ormyridae and Pteromalidae (Wang et al. 2015a; Burks et al. 2022). They exploit the mutualism between fig trees and their pollinator and undermine the reproductive success of their host plant by reducing pollen dispersal and destroying ovules and seeds (Suleman et al. 2013; van Noort et al. 2013). As with the pollinator, many of these species display sexual dimorphism where females have fully developed eyes and wings in order to disperse in search of new host plants, whereas their males are often wingless, with smaller eyes and dwell their entire live inside the fig (Weiblen 2002; Zhou et al. 2012).

Following the description of the pollinator *E. verticillata* (Waterston, 1921), Ishii (1934) described six non-pollinating fig wasps from Japan. Years later, extended surveys of ornamental individuals of *F. microcarpa* from Florida revealed that four fig wasp species had managed to follow their host plant outside their native range for the first time (Nadel et al. 1992). Chen et al. (1999) described ten additional species from Taiwan and finally Wang et al. (2015a) created a list of a total of 32 morphospecies and species associated with *F. microcarpa*. Recently, Demetriou et al. (2023) reported eleven fig wasp species from the island of Cyprus, even reporting on finding a presumably new undescribed species belonging to Epichrysomallidae. Regarding Greece, Compton (1989) reported *Odontofroggatia galili* Wiebes, 1980 from the island of Symi as the first introduced fig wasp species in Europe. Material samplings from Rhodes and Symi nearly three decades after, revealed a total of seven alien fig wasp species (Wang et al. 2015a), suggesting that new species have been colonising rapidly in the intervening years. To date, no structured survey has been ever carried out at a mainland country level regarding the wasp fauna of *F. microcarpa*. Herein, we present the first detailed study on the fig wasp species related to *F. microcarpa* in Greece reporting on a total of 13 associated species.

Materials and methods

The only publications dealing with the fig wasp fauna of Greece are limited to Compton (1989), Wang et al. (2015a) and Wang et al. (2015b), where sampling was mainly restricted to the south-eastern Aegean islands of Rhodes and Symi. In order to bridge the knowledge gap, sampling was carried out throughout Greece, with an emphasis on its southern part, where *F. microcarpa* is more widespread. Since distributional records for *F. microcarpa* were not found in the scientific literature [except for Galanos (2015) - reporting for the first time on spontaneous plants near parent plants in urban areas of Rhodes], before visiting areas where host trees might be present, Google Maps, Google Street view, social media and citizen-science platforms (such as iNaturalist) were utilised. Starting in early 2021, samples were obtained in Attica (Att), Central Greece (CG), Crete (Cr), Cyclades (Cy), Dodecanese (Do), Ionian islands (Iis) and Peloponnese (Pel) (Fig. 1, Table 2, Suppl. material 2).

Monoecious figs development follows a series of five phases (A-E), with pollinators laying their eggs at B-phase, developing during C-phase and emerging during D-phase, while NPFW mainly oviposit during B or C-phase (a very few oviposit at A phase) and their offspring emerge at the same time with those of the pollinator (Galil and Eisikowitch 1968a; Wang et al. 2015a). As such, upon locating the fig tree crops, figs in late C to early D phase were collected, since these stages are optimal for obtaining adult wasps (Galil and Eisikowitch 1968a; Wang 2014; Wang et al. 2015a). During each collecting event, figs were put in sealed polyethylene bags and subsequently stored in 90° ethanol. Following Wang et al. (2015a), after removed from ethanol, collected figs were put in plastic boxes filled with water for 10 minutes and each fig was divided into four parts for further examination.

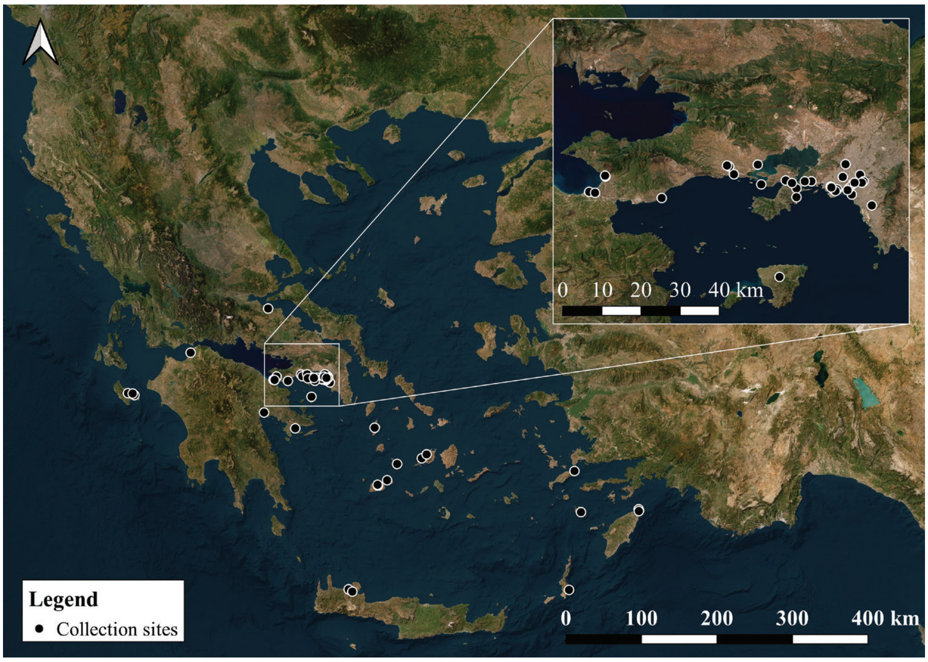

Figure 1. Collections of *Ficus microcarpa* figs.

Specimen rearing, storage and identification

Reared fig wasps that emerged before the figs' storage, were stored in 70° and 90° ethanol, for identification under a stereomicroscope and molecular treatment. A total of 7,292 figs from 58 localities were collected for this study. Dissected figs were observed under a stereomicroscope and observed galls were carefully opened with entomological forceps for the extraction of the wasps. In a similar manner, extracted samples were put in ethanol for future morphological or molecular examination. Specimens used for identification were either air-dried or dried using hexamethyldisilazane (HMDS) (Heraty and Hawks 1998) and mounted on rectangular paper cards. Identification was carried out following a combination of keys and descriptions (Ishii 1934; Beardsley 1998; Chen et al. 1999; Feng and Huang 2010; Ma et al. 2013; Wang et al. 2016; Xiao et al. 2021; van Noort and Rasplus 2022; Burks et al. 2022).

Molecular analysis

To ensure that all specimens are unambiguously assigned to a particular *Philotrypesis* species, besides using traditional morphological features, we also performed a molecular identification. After the PCR amplification of a fragment of the mitochondrial DNA (mtDNA) gene of Cytochrome c Oxidase Subunit I (COX1), the generated sequences, together with all available congeneric COX1 sequences available in GenBank, were analysed within a phylogenetic framework aiming to assess the relationship of the specimens of this study to the other *Philotrypesis* species.

DNA extraction and PCR amplification

Total genomic DNA from *Philotrypesis* individuals preserved in 100% ethanol was extracted using the CTAB (hexadecyl-trimethyl-ammonium bromide) protocol, as described in Parmakelis et al. (2005). In total, we extracted the DNA from 24 specimens. The whole body of the individuals was used in the extraction. However, specimens belonging to the same series with specimens used for DNA extraction were dried with HMDS and card mounted, allowing future morphological comparisons. PCR was carried out to amplify a fragment of the mitochondrial gene COX1 using the primer pair mt6 (C1-J-1718) and Nancy (C1-N-2191) (Simon et al. 1994). The total reaction volume was 25 μl, where 2 μl of template DNA was mixed with 0.2 mM dNTP's, 0.4 μM of each primer and 0.5 units of Taq polymerase and 3.5 mM $MgCl_2$. The reaction consisted of an initial denaturation step of 95 °C for 3 min, followed by 39 cycles of 15 sec at 94 °C, 1 min at 40 °C and 1.5 min at 72 °C. The cycling ended with a 10 min extension step at 72 °C. The PCR products were purified using the NucleoSpin Gel and PCR Clean-Up Kit (Macherey-Nagel). The purified PCR products were sequenced using the BigDye™ Terminator v.3.1 Cycle Sequencing Kit, utilising an automated sequencer at the CeMIA sequencing facility (CEMIA S.A., Greece). Both strands were sequenced and the primers used in the sequencing reactions were the same as in the amplification steps. Sequences were deposited in GenBank under the accession numbers PQ897148, PQ897149, PQ897150, PQ897151, PQ897152, PQ897153, PQ897154, PQ897155, PQ897156, PQ897157, PQ897158, PQ897159, PQ897160, PQ897161, PQ897162, PQ897163, PQ897164 and PQ897165.

Phylogenetic analysis

Along with the 18 sequences generated within this study, in the phylogenetic analysis, we used 276 COX1 sequences of *Philotrypesis* species that were available in GenBank. In addition, we used sequences of *Sycoscapter* spp. and *Walkerella microcarpae* Bouček, 1993 as outgroup taxa. Details of the origin of specimens used in the study and the accession numbers of sequences retrieved from GenBank are provided on Suppl. material 1. The generated *Philotrypesis* sequences were edited using CodonCode Aligner v. 9.0.1. The same software was used in the alignment of the sequences used in the phylogenetic analysis. Prior to the phylogenetic analysis, we used IQ-TREE v. 2.2.0 (Minh et al. 2020) in order to evaluate the best fitting nucleotide substitution model for the data at hand. The Bayesian Information Criterion (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017) was used to identify the best fitting model. The model selected and applied in the analysis was the GTR+I+G. MrBayes v. 3.2.7 (Ronquist and Huelsenbeck 2003) was used to perform Bayesian Inference analysis (BI). We set the parameters and priors, based on the substitution model implemented. The number of generations was set to 2×10^6 and two independent runs with four chains were performed. A consensus tree was constructed after discarding 25% of the generated trees as burn-in.

Results

Molecular and morphological identification of *Philotrypesis* specimens

The results of the phylogenetic analysis performed are presented in Fig. 2. It can be seen that all currently known *Philotrypesis* species for which available data existed in GenBank, were recovered as monophyletic groups. An exception to this is the species *Philotrypesis emeryi* Grandi, 1926, for which the respective sequences did

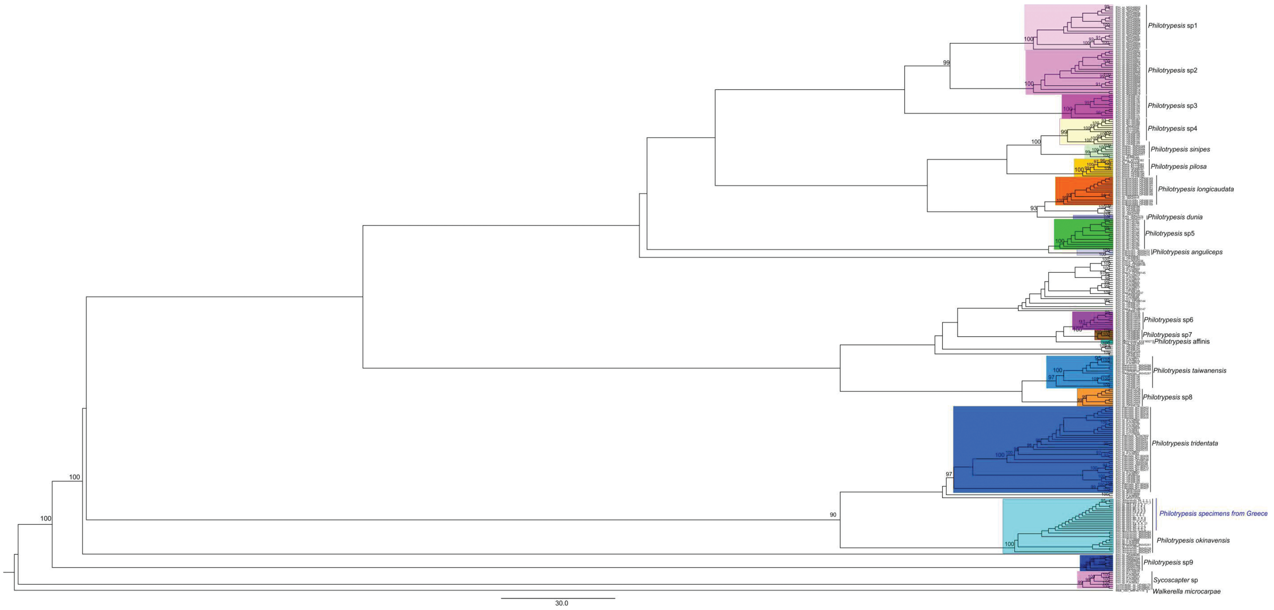

Figure 2. 50% majority rule consensus tree of the Bayesian Inference (BI) analysis. Values at nodes indicate the posterior probability (percent values) support of nodes. Only values above 90% are shown. *Philotrypesis* species for which sequence data were available in GenBank are indicated. Clades not corresponding to known *Philotrypesis* species are marked as sp1, sp2 etc. The specimens of *Philotrypesis* originating from Greece are indicated with the vertical blue bar.

not form a single clade. This could be due to several reasons. Misidentification of specimens deposited in GenBank is only one of them. However, the phylogenetic results point to the need for a taxonomic revision for this species. At this point, it should be mentioned that there are many cases where monophyletic groups are formed by several unassigned *Philotrypesis* lineages. This is typical in cases where cryptic speciation is present amongst morphologically indistinguishable species.

Regarding the affinity of the *Philotrypesis* specimens of this study, it seems that they are firmly placed within the *P. okinavensis* lineage (*sensu* Zhou et al. (2012)) that forms a well-supported monophyletic group. Especially regarding the specimens from Rhodes, they are clearly phylogenetically distinct from all instances of *P. emeryi* appearing on the tree. Although *P. emeryi* was first reported from both Rhodes and Symi (Wang et al. 2015a), morphological and molecular examination of our specimens from the island of Rhodes (Suppl. material 1) allowed us to exclude it from this list. Therefore, the reports of this species in Wang et al. (2015a) are misidentifications of *Philotrypesis okinavensis* Ishii, 1934.

The revised checklist of fig wasps associated with *F. microcarpa* in Greece includes thirteen (13) species, distributed in five families (Table 1, Figs 4, 5). A total of six species are reported for the first time from Greece: (i.e. *Eufroggattisca okinavensis*, *Micranisa degastris*, *Odontofroggatia quinifuniculus*, *Philotrypesis okinavensis*, *Philotrypesis taiwanensis*, *Sycophila curta*) and two of them (i.e. *O. quinifuniculus*, *S. curta*) are recorded outside their native range for the first time.

Species occurrence/frequency data

A total of 21457 fig wasps were sampled within this survey. Amongst the collected fig wasp species, *E. verticillata* was the most abundant (11.537 individuals), followed by *O. galili* (3.827), *M. bicolor* (1.840) and *W. microcarpae* (1.687) (Tables 2, 3). The pollinator was also widely distributed (82.7% prevalence), followed by

Table 1. Updated checklist of fig wasps associated with *F. microcarpa* in Greece, including their trophic regime.

No	Family	Subfamily	Species	Abbreviation	Trophic regime	Reference
1	Agaonidae	Agaoninae	*Eupristina verticillata* Waterston,1921	EV	ovule galler	Wang et al. (2015a)
2	Epichrysomallidae		*Eufroggattisca okinavensis* Ishii, 1934	EO	unknown	Present study
3			*Meselatus bicolor* Chen, 1999	MB	ovule galler	Wang et al. (2015a)
4			*Odontofroggatia galili* Wiebes, 1980	OG	ovule galler	Compton (1989)
5			*Odontofroggatia ishii* Wiebes, 1980	OI	ovule galler	Wang et al. (2015a)
6			*Odontofroggatia quinifuniculus* Feng & Huang, 2010	OQ	ovule galler	Present study
7	Eurytomidae	Eurytominae	*Sycophila curta* Chen, 1999	SC	parasitoid	Present study
8			*Sycophila maculafacies* Chen, 1999	SM	parasitoid	Wang et al. (2015a)
9	Ormyridae		*Ormyrus microcarpae* Askew & Koutsoukos, 2024	OR	parasitoid	Koutsoukos et al. (2024)
10	Pteromalidae	Pteromalinae	*Micranisa degastris* Chen, 1999	MD	ovule galler	Present study
11			*Philotrypesis okinavensis* Ishii, 1934	PO	parasitoid	Wang et al. (2015a)
12			*Philotrypesis taiwanensis* Chen, 1999	PT	seed eater	Present study
13			*Walkerella microcarpae* Bouček, 1993	WM	ovule galler	Wang et al. (2015a)

the phytophagous gallers *O. galili* (70.6%), *W. microcarpae* (70.6%) and *O. ishii* (43.1%) (Suppl. material 3). The most species-rich collection sites were located within Attica, Peloponnese and South Aegean (Cyclades and Dodecanese Islands) areas (10 species), followed by Crete (7 species). The most widely distributed species were *O. galili* (7 administrative divisions), followed by the pollinator *E. verticillata* (6 administrative divisions) and *M. bicolor*, *O. ishii* and *W. microcarpae* (5 administrative divisions) (Tables 2, 3).

Amongst the three time-intervals, one species was reported in 1989, six more in 2014 and seven more again during the last few years (Koutsoukos et al. 2024; present study). Regarding the ecology and trophic regimes of alien fig wasps, eight out of the thirteen collected species are phytophagous ovule gallers (including *E. verticillata*), while one is a seed eater (*P. taiwanensis*). The remaining four species are parasitoids. Namely, *Sycophila* spp. are parasitoids of *Odontofroggatia* spp., *P. okinavensis* is a parasitoid of *W. microcarpae*, while *Ormyrus microcarpae* is most likely a parasitoid of *M. bicolor* (Wang et al. 2015a; Koutsoukos et al. 2024).

Discussion

Distribution, abundance and diversity

We have presented the first survey of fig wasps associated with *F. microcarpa* at a country level in Greece. With six species presented as new records for the country, the fig wasp fauna has almost doubled since Compton (1989) and Wang et al. (2015a), currently including 13 alien species. At least 38 species are known to be associated with *F. microcarpa* in its native and introduced ranges, of which 16 have been reported outside their native range at least once, while the rest, to our knowledge, are restricted to their natural distribution (van Noort and Rasplus 2010; Li et al. 2013; Wang 2014; Wang et al. 2015a; van Noort and Rasplus 2022; Demetriou et al. 2023). Amongst the species collected, *O. quinifuniculus*, and *S. curta* were found outside their natural distribution for the first time. Interestingly, *O. microcarpae* was recently detected and described from Greece and Cyprus (Koutsoukos et al. 2024) and its precise native range remains unknown.

Table 2. Sampled localities and their species composition.

Adm. division	Site	Coordinates	Date(s)	Number of crops	Number of figs	Species present
ATT	Argiroupoli	37°54'32.4"N, 23°45'03.6"E	22.x.2021	1	23	OG, WM, EV, MB, SM
ATT	Athens Centre	37°58'45.7"N, 23°42'48.3"E	vii.2021	3	20	OG, EV
ATT	Egaleo, PADA	38°00'15.1"N, 23°40'32.9"E	iv-ix.2021	16	392	OG, OI, OQ, WM, PO, EV, MB, OR, SM, SC
ATT	Egina, Agios Nektarios	37°44'49.7"N, 23°29'01.5"E	7.xii.2021	1	37	OG, PO, EV
ATT	Faliro, Delta	37°56'03.6"N, 23°41'37.1"E	iv.-ix.2021	2	351	OG, OI, WM, EV, MB, SM, SC
ATT	Kallithea, Ilissos	37°56'58.7"N, 23°41'10.4"E	28.viii.2021	1	10	EV
ATT	Kallithea, Park 1	37°57'30.7"N, 23°41'48.0"E	28.viii.2021	1	17	OG, WM, EV
ATT	Kallithea, Park 2	37°57'41.0"N, 23°42'07.1"E	28.viii.2021	1	72	OG, WM, EV
ATT	Megara	37°59'46.0"N, 23°20'40.1"E	v-xi.2021	4	27	OG, EV
ATT	Megara, Exo Vrisi	37°59'58.3"N, 23°20'00.3"E	31.x.2021	1	306	OG, OI, WM, PO, EO, EV, MB, SM
ATT	Megara, Pachi	37°58'51.6"N, 23°21'12.6"E	4.vii.2021	1	63	WM, EV
ATT	Moschato	37°56'42.9"N, 23°40'49.6"E	v-viii.2021	1	84	OG, OI, WM, EV
ATT	Nea Peramos	38°00'04.3"N, 23°25'14.6"E	iv-xi.2021	9	1080	OG, OI, WM, PO, EO, EV, MB, OR, SM
ATT	Neos Kosmos, Fix	37°57'52.8"N, 23°43'39.3"E	28.viii.2021	1	9	EV
ATT	Neos Kosmos, Syngrou	37°57'43.1"N, 23°43'20.7"E	28.viii.2021	1	2	OG, WM, EV, SM
ATT	Perama, Centre	37°57'48.1"N, 23°34'31.2"E	iv-x.2021	4	425	OG, OI, WM, PO, EV, MB, OR, SM
ATT	Perama, Port	37°57'51.2"N, 23°33'31.0"E	v-x.2021	2	123	OG, OI, WM, PO, EV, MB, SM, SC
ATT	Piraeus, Port	37°56'43.9"N, 23°38'27.6"E	viii-x.2021	1	764	OG, OI, WM, PO, EO, EV, MB, OR, SM, SC
ATT	Piraeus, Arch. Museum	37°56'49.0"N, 23°39'02.6"E	28.viii.2021	1	60	EV
ATT	Piraeus, Korai Square	37°56'31.6"N, 23°38'50.1"E	viii-ix.2021	3	74	OG, WM, EV
ATT	Piraeus, Sfaktirias Square	37°57'07.9"N, 23°37'58.7"E	5.x.2021	1	15	OG, EV
ATT	Piraeus, Vourlon Square	37°56'59.0"N, 23°38"01.1"E	5.x.2021	2	7	EV
ATT	Renti	37°58'28.1"N, 23°40'02.8"E	25.ix.2021	1	17	OG, WM, EV, MB
ATT	Salamina, Agia Kyriaki	37°58'05.9"N, 23°30'08.4"E	v-viii.2021	2	353	OG, OI, WM, EO, EV, MB
ATT	Salamina, Ambelakia	37°56'54.1"N, 23°32'00.9"E	9.v.2021	2	15	OG, WM, EV
ATT	Salamina, Iliakti	37°57'25.8"N, 23°25'54.5"E	27.ix.2021	1	47	OG, EV
ATT	Salamina, Selinia	37°55'44.3"N, 23°32'06.0"E	iv-vi.2021	1	77	OG, OI, WM, EV, MB
ATT	Salamina, Sinikismos	37°57'37.5"N, 23°31'14.5"E	23.viii.2021	1	178	OG, WM, EV

Adm. division	Site	Coordinates	Date(s)	Number of crops	Number of figs	Species present
ATT	Tauros	37°57'37.1"N, 23°41'58.8"E	28.viii.2021	1	72	EV
CG	Agios Konstantinos	38°45'20.5"N, 22°53'46.1"E	2.x.2021	1	17	EV
Cr	Chania, Municipal Garden	35°30'42.1"N, 24°01'30.7"E	14. xi.2021	4	11	OG, OI, WM, PO, EV, MB, SM
Cr	Chania, Municipal Market	35°30'51.5"N, 24°01'14.8"E	14. xi.2021	9	23	OG, OI, WM, PO, EV, MB, SM
Cr	Chania, Souda	35°29'14.3"N, 24°04'37.0"E	14. xi.2021	1	117	OG, OI, WM, PO, EV, SM, SC
Cy	Antiparos, Chora	37°02'25.1"N, 25°04'51.5"E	18.ix.2021	1	36	OG, WM, PO, EV, SM
Cy	Kimolos, Psathi	36°47'14.4"N, 24°34'42.6"E	3.ix.2021	1	188	OG, OI, WM, PO, EV, MB,
Cy	Kithnos, Merichas	37°23'31.8"N, 24°23'51.4"E	27.vi.2021	1	26	OG, EV
Cy	Milos, Adamas	36°43'27.5"N, 24°26'37.4"E	4.ix.2021	1	183	OG, OI, WM, PO, EV, MB, OR, SM, SC
Cy	Milos Katifora	36°44'07.4"N, 24°26'30.9"E	5.ix.2021	1	134	OG, OI, WM, PO, EV, SM
Cy	Paros, Paroikia	37°05'07.3"N, 25°08'56.1"E	18.ix.2021	3	438	OG, OI, WM, EV, SM, SC
Cy	Sifnos, Artemonas	36°58'25.9"N, 24°43'24.1"E	viii.2021	1	52	EV
Do	Karpathos, Pigadia	35°30'29.5"N, 27°12'39.5"E	viii.2021	1	40	WM, EV
Do	Kos	36°53'36.2"N, 27°17'20.8"E	vi.2021	1	12	EV
Do	Rhodes, Agios Frangiskos	36°26'21.3"N, 28°13'28.9"E	viii.2021	1	29	EV
Do	Rhodes, Ethinkis Antistasis	36°26'00.3"N, 28°13'26.2"E	viii.2021	1	71	OG, OI, WM, MD, PO, PT, EO, EV, MB, SM, SC
Do	Rhodes, Rodiaki Epavli	36°26'52.6"N, 28°13'08.2"E	viii.2021	1	191	OG, OI, WM, PO, EV
Do	Rhodes, Rodini Park	36°25'37.2"N, 28°13'16.7"E	viii.2021	1	87	OG, WM, PO, EV
Do	Tilos	36°24'58.2"N, 27°23'06.8"E	vi.2021	5	355	OG, OI, WM, EV
Iis	Zakynthos Ag. Kirikos	37°47'15.1"N, 20°49'41.6"E	10.iv.2022	1	3	OG, EV
Iis	Zakynthos Argasi	37°45'54.6"N, 20°55'20.1"E	9.iv.2022	1	7	OG, WM
Iis	Zakynthos Chora	37°46'55.0"N, 20°53'50.0"E	10.iv.2022	3	26	OG, OI, WM
PEL	Agioi Theodoroi	37°55'33.6"N, 23°08'34.8"E	iii-ix.2021	4	148	OG, OI, WM, PO, EO, EV, MB, OR, SM, SC
PEL	Ermioni	37°23'08.2"N, 23°14'54.6"E	vi.2021	1	15	OG, WM, EV, SM
PEL	Loutraki, Park	37°58'25.1"N, 22°58'45.2"E	vi-ix.2021	2	100	OG, OI, WM, PO, EV
PEL	Loutraki, Spiritual Centre	37°58'36.4"N, 22°58'43.3"E	vi-ix.2021	2	80	OG, OI, WM, PO, EV, SM, SC
PEL	Korinthos Centre	37°56'23.7"N, 22°56'00.7"E	25.vii.2021	1	61	OI, WM, EV
PEL	Korinthos South Town	37°56'18.8"N, 22°56'59.0"E	25.vii.2021	1	16	WM, EV
PEL	Nafplio	37°33'59.9"N, 22°48'01.2"E	v-xi.2021	4	74	OG, OI, WM, PO, EO, EV, MB OR, SM
PEL	Patra	38°14'56.5"N, 21°44'06.1"E	8.iv.2022	1	32	OG, EV

Table 3. Number of occupied administrative divisions, year of first published record, total number of specimens and percentage for each fig wasp species recorded from Greece.

Species	Year of first record	Number of adm. divisions occupied	Total number of specimens	Percentage
Eupristina verticillata	2014	6	11537	53.76%
Eufroggattisca okinavensis	2023	2	82	0.003%
Meselatus bicolor	2014	5	1840	0.085%
Odontofroggatia galili	1989	7	3827	0.1783%
Odontofroggatia ishii	2014	5	984	0.045%
Odontofroggatia quinifuniculus	2023	1	4	0.0001%
Sycophila curta	2023	4	59	0.002%
Sycophila maculafacies	2014	4	619	0.028%
Ormyrus microcarpae	2023	3	239	0.011%
Micranisa degastris	2023	1	9	0.0004%
Philotrypesis okinavensis	2014	4	553	0.025%
Philotrypesis taiwanensis	2023	1	17	0.0007%
Walkerella microcarpae	2014	5	1687	0.078%

Recently, and increasing number of fig wasp species have been found outside their native range, while there is lack of knowledge regarding the extend of their distribution in their putative native ranges. These include *Acophila microcarpa* Chen, 1999 (UAE), *Eufroggattisca okinavensis* Ishii, 1934 (Cyprus), *Odontofroggatia corneri* Wiebes, 1980 (South Africa) and *Sycophila petiolata* Chen, 1999 (Cyprus) (van Noort and Rasplus 2010; van Noort et al. 2013; Demetriou et al. 2023). The application of molecular tools has proved to be crucial on the research of fig wasp species that are very closely related as shown in this study by the detection of the erroneous assignment of *P. okinavensis* specimens from Greece to *P. emeryi* (Wang et al. 2015a). *Philotrypesis emeryi* has been also reported from neighbouring Turkiye (Doganlar 2012). Nevertheless, given that only *P. okinavensis* occurs in Greece (and by extension into the SE Aegean, which is in close proximity with the neighbouring country) and Cyprus, records from Turkiye should be re-evaluated using both morphological and molecular tools in order to rule out possible misidentifications in Doganlar (2012). Future application of molecular tools alongside morphological examination of specimens is expected to reveal further cryptic or new species and, as such, the number of alien fig wasps could further rise (Sun et al. 2011; Wang et al. 2015a; J-Y Rasplus, pers. comm.). To support this claim, in the phylogenetic analysis of *Philotrypesis* performed in this study, already a number of undescribed *Philotrypesis* lineages have been indicated (Fig. 2).

The presence of the fig wasp species in Greece is likely to be the result of recent introductions. This can be supported by the number of species recorded in the area (Dodecanese Islands) where three different sampling attempts were made at different time intervals (Wang et al. 2015a). Starting with the initial detection of *Odontofroggatia galili* from the island of Symi (Compton 1989), samples from the islands of Rhodes and Symi twenty-five years later increased the number of fig wasp species to seven (Wang et al. 2015a), eventually rising to thirteen with the addition of six species in the present study. Interestingly, most of these species (ten out of thirteen) are present in the Dodecanese Islands. This trend seems to follow globalisation, international trade and the translocation of exotic plants around the world (Mack et al. 2000; Wang et al. 2015a). Nevertheless, the possibility that these species could have been present from the earliest introductions of *F. microcarpa* to Greece cannot be completely ruled out, given this is the first extensive sampling effort carried out on a national level. Wang et al. (2015a) discussed how the secondary spread of different fig wasp species throughout the Mediterranean could have already taken place or is likely to take place in the future, either naturally or via human mediation. Some of the fig wasps associated with *F. microcarpa* could have reached the country from already invaded territories, such as Cyprus and Turkiye, where a significant number of species are present (Doganlar 2012; Wang et al. 2015a; Demetriou et al. 2023), with the assistance of air currents (van Noort and Rasplus 2010). It is possible that some of these species have managed to spread throughout the Aegean in the same manner. After all, air-borne movement by fig wasps has already been recorded to exceed distances of up to 160 km in the Namib Desert (Ahmed et al. 2009).

Invasiveness and impact of *Ficus microcarpa*

As mentioned above, *F. microcarpa* is an ornamental plant with a wide distribution beyond its natural range. Followed and assisted by its pollinator *E. verticillata*, it has managed to establish and even become invasive in some areas (Malta, Hawaii and many southern parts of the USA) (Wang et al. 2015b; Compton et al. 2018). In Greece, cases of germination and development of small seedlings have already been reported on the medieval walls of Rhodes (Galanos 2015). Similar cases of seedlings developing on walls were observed in the port of Piraeus near fig trees (Fig. 3a, Table 4). Due to its resistance to salinity, a well-developed *F. microcarpa* shrub was also observed on the dock of Agios Nikolaos (Crete) with its base standing just a few centimetres above sea level (Fig. 3b). These observations reinforce our concerns that *F. microcarpa* may eventually manage to escape urban habitats into semi-natural or even natural habitats, affecting native plant diversity. This is of particular importance in the Dodecanese and other Aegean Islands, where the establishment of an alien plant may disproportionally affect native island flora (Ramírez and Montero 1988; Starr et al. 2003; Reaser et al. 2007; Hulme et al. 2008; Russell et al. 2017). The socioeconomic and human-health risks related to the ripe fallen figs are also worth discussing. Ripe figs falling to the ground have been mentioned to form a slippery amorphous mass, which can not only attract harmful insects (i.e. flies), but also endanger pedestrians or damage property such as parked cars (Fig. 3c, d) (Ramírez and Montero 1988; Beardsley 1998; Tsintides et al. 2002; Demetriou et al. 2023).

Table 4. List of sites in which seedlings of *F. microcarpa* were observed, in Greece.

Administrative region/locality	Coordinates	Date	Habitat	Type of infrastructure seedlings were found on	Reference
Dodecanese/Rhodos town/Rodiaki epavli	36°26'53"N, 28°13'10"E	21.xi.2014	Urbanized	In crevices of walls	Galanos (2015)
Dodecanese/Rhodos town	36°26'23"N, 28°13'25"E	11.i.2015	Urbanized	Medieval castle walls	Galanos (2015)
Dodecanese/Rhodos town	36°26'46"N, 28°13'41"E	18.i.2015	Urbanized	Medieval castle walls	Galanos (2015)
Attica/Piraeus port	37°56'44.2"N, 23°38'27.9"E	25.vi.2021	Urbanized	Marble wall	Present study
Crete/Agios Nikolaos town	35°11'25.3"N, 25°43'05.1"E	24.viii.2022	Urbanized	Sea wall	Present study
Peloponnese/Xylokastro	38°04'40.8"N, 22°37'40.8"E	8.x.2022	Urbanized	Sidewalk	Present study
Peloponnese/Nafplio	37°33'57.0"N, 22°47'58.5"E	19.xi.2023	Urbanized	On *Phoenix* sp.	Present study

Figure 3. **a** *Ficus microcarpa* small tree, in Agrios Nikolaos port, Crete **b** *F. microcarpa* seedling in Piraeus port **c, d** Ripe figs under *F. microcarpa* trees in Heraklion, Crete.

Aiming to mitigate the impact and the possibility of *F. microcarpa* becoming invasive, Wang et al. (2015b) investigated the use of gall-inducing fig wasps as biological control agents against the pollinator *E. verticillata*. While *O. galili* is the most widespread fig wasp species after the pollinator in its invaded areas, its role on the reduction of pollination was not deemed significant (Wang et al. 2015b). In contrast, *M. bicolor* creates galls that nearly always prevent pollination and seed production (Wang et al. 2015b). While this species was only known from the Dodecanese Islands at the time, Demetriou et al. (2023), as well as the present study, show that it has a remarkable distribution in both Greece and Cyprus. The species' extended distribution could enhance efforts to study its potential in the biological control of its host-plant, although the discovery of its parasitoid *O. microcarpae* could be a hindering factor for such schemes (Koutsoukos et al. 2024). Nevertheless, given the large number of fig wasp species associated with *F. microcarpa*, newly-arrived species may prove equally effective or better than *M. bicolor*.

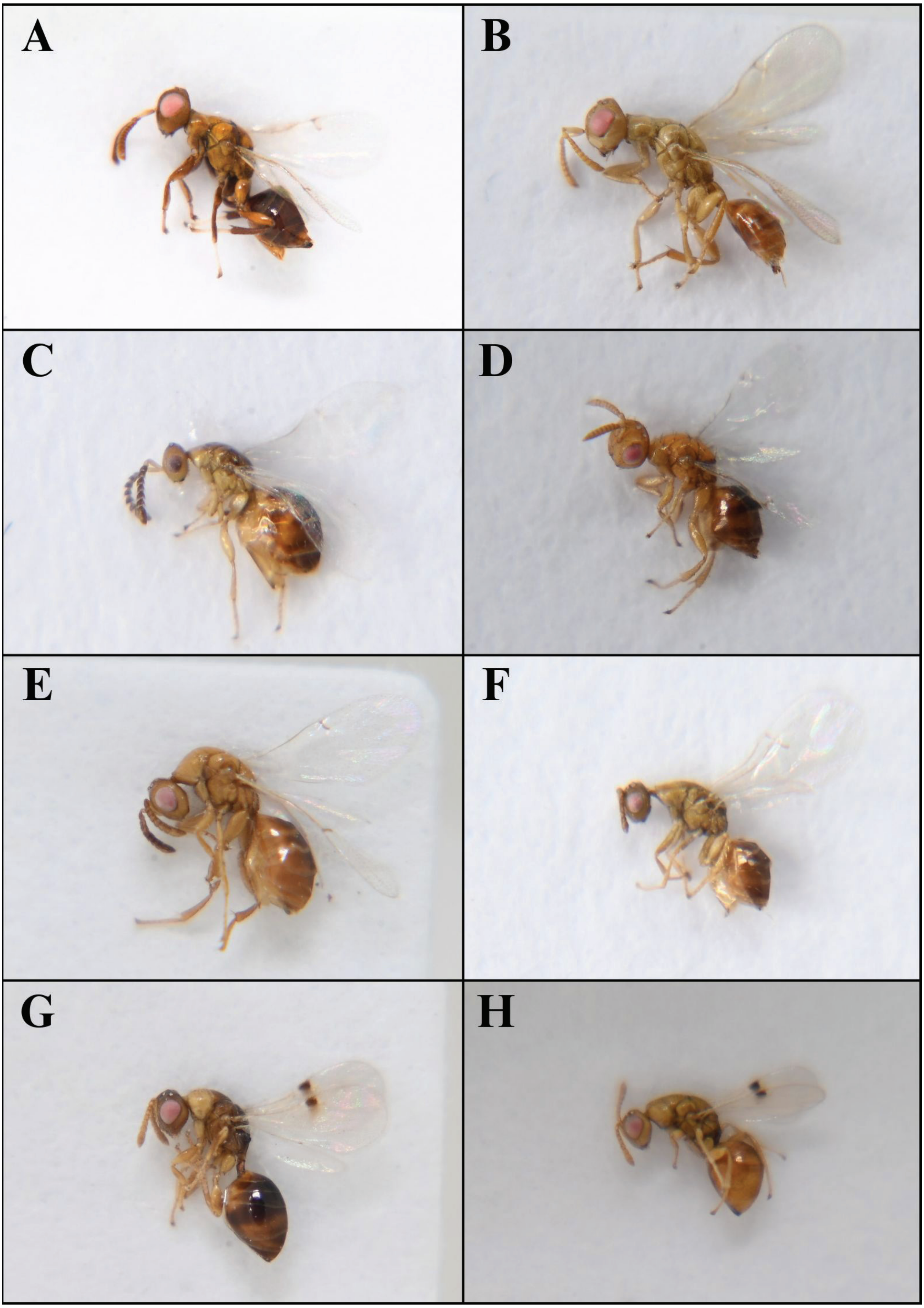

Figure 4. Species reared from *F. microcarpa* figs during this study **A, B** *Eufroggattisca okinavensis*, female and male **C** *Odontofroggatia galili*, female **D** *Meselatus bicolor*, female **E** *Odontofroggatia ishii*, female **F** *Odontofroggatia quinifuniculus*, female **G** *Sycophila maculafacies*, female **H** *Sycophila curta*, female.

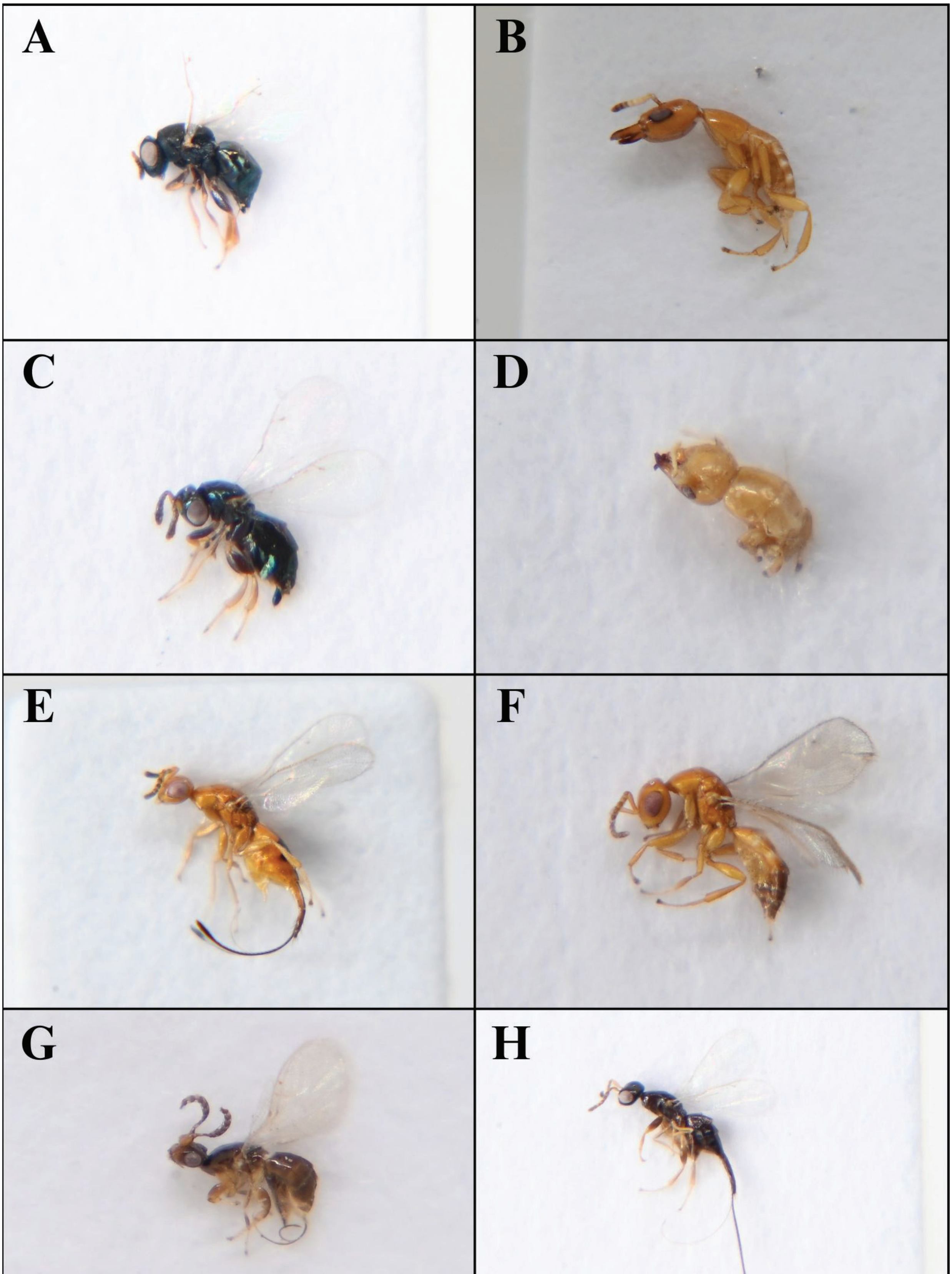

Figure 5. Species reared from F. microcarpa figs during this study **A, B** *Walkerella microcarpae*, female and male **C, D** *Micranisa degastris* **E, F** *Philotrypesis okinavensis*, female, male **G** *Eupristina verticillata* **H** *Philotrypesis taiwanensis*, female.

As remarked above, *M. bicolor* is the NPFW with the highest impact on both the pollinator and the tree, even though it does not have the same abundance and prevalence as *O. galili*. *Odontofroggatia* species, *W. microcarpae*, *P. taiwanensis* and *M. degastris* can also negatively affect *F. microcarpa* by interacting with the pollinator, although insignificantly, when compared with *M. bicolor*. Nevertheless, the widespread and high abundance of species, such as *O. galili* and *W. microcarpae*, could be auxiliary to efforts for limiting *E. verticillata*. Other NPFW species present in Greece include parasitoids that attack the aforementioned species that are competitive to the pollinator and, as such, are beneficiary to *E. verticillata* and the reproduction and expansion of *F. microcarpa*. These include both *Sycophila* species (parasitoids attacking *Odontofroggatia* spp.) and most notably the newly-discovered *O. microcarpae* that attacks *M. bicolor*. Species with an ambiguous role include *P. okinavensis*, which parasitises both *W. microcarpae* and the pollinator (Compton et al. 2018) and *E. okinavensis*, which is found in *M. bicolor* galls, although whether it is a parasitoid or an inquiline is yet unclear.

Given the widest distribution (82.7% prevalence) and highest percentage (53.76%) amongst all species collected, *E. verticillata* will continue to assist its host-plant to reproduce. As such, it is distinctly possible that cases of germination and development of seedlings will multiply and all adverse socioeconomic impacts associated with *F. microcarpa* will persist. Taking into account the rich fig wasp fauna associated with *F. microcarpa* in Greece, future research could shed further light on the complex trophic webs within figs and how these are affecting *E. verticillata*. This could possibly serve as the baseline for monitoring schemes in order to prevent *F. microcarpa* establishing in native habitats and becoming invasive.

Conclusions

A total of 13 alien fig wasp species have been recorded from Greece, with their number almost doubling since previous studies (Wang 2014; Wang et al. 2015a; Wang et al. 2015b). Nevertheless, as new alien species are constantly being discovered outside their native range, including species associated with *Ficus* spp. (Beardsley and Rasplus 2001; Koutsoukos et al. 2024), their numbers are predicted to increase even further. These species are mostly represented in the Aegean Islands, where their host plant is more common due to warmer climatic conditions.

As earlier discussed, new records are hypothesised to represent newly-introduced species; nevertheless, this statement is not definitive. As such, wider-scale sampling is necessary to assess the biodiversity of fig wasps on a national level. Furthermore, the application of molecular tools is planned to investigate gene flow and the genetic diversity, especially between island populations. As new additions to the checklist are anticipated, it is important to investigate the introduction pathways and means of spread of alien fig wasps, as well as their interplay with *F. microcarpa*, ensuring the plant will not manage to escape cultivation.

The reported displacement of native flora by *F. microcarpa* in invaded parts of the world is problematic (Wang et al. 2015a). Seedlings both in Greece and Cyprus have been observed in urban habitats, with their spread being assisted by pollination of the figs by *E. verticillata* and the subsequent ingestion of ripe fruits and excretion of the seeds by birds (Galanos 2015; Demetriou et al. 2023; present study). In Cyprus, seedlings in the Limassol city centre were found in areas with sufficient irrigation (under rainspouts) and covered in bird droppings.

This shows that not all available natural habitats could be potentially colonised by *F. microcarpa*, especially in the arid Mediterranean islands. Nevertheless, habitats with sufficient moisture or water content visited by birds, such as riparian vegetation and other inland waterbodies, already hosting a large number of naturalised alien plants (Arianoutsou et al. 2010), could be monitored in the future.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

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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

DNA barcoding specimen codes, accession numbers and specimen details for representatives of the genus *Philotrypesis*

Authors: Evangelos Koutsoukos

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.98.139650.suppl1

Supplementary material 2

Detailed number of specimens per species from each sampled locality

Authors: Evangelos Koutsoukos

Data type: xlsx

Link: https://doi.org/10.3897/neobiota.98.139650.suppl2

Supplementary material 3

Presence and prevalence of fig wasp species

Authors: Evangelos Koutsoukos

Data type: xlsx

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Review Article

The invasive mysid *Hemimysis anomala*: an up-to-date review of its biology, ecology, distribution and ecological impacts

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Abstract

The "bloody-red shrimp" species *Hemimysis anomala*, native to the Ponto-Caspian Region, has spread rapidly and adapted over recent decades to new aquatic ecosystems worldwide, causing many ecological alterations. The reported impacts associated with this invasion are numerous, including overall food web structure modifications. This review focuses on *H. anomala*, examining its biology, ecology, distribution and ecological impacts. The species' rapid success is attributed to its high fecundity, fast growth rates and broad adaptability to various habitats. Recent observations suggest that its distribution may expand further, especially in Western European lakes, reservoirs and the Laurentian Great Lakes. We present insights into its habitat, seasonal dynamics and influence on trophic interactions within native zooplankton communities. Additionally, we discuss methods used to study *H. anomala*, including citizen-science initiatives that enhance data collection and community engagement. The objective of this research is to provide up-to-date pieces of information on *H. anomala*'s ecology, including new data on its habitat preferences collected through scientific diving and participatory research. By characterising the spatial and temporal variation in its occurrence and abundance, we identify key environmental and biotic factors that may limit or support its further spread. We also highlight knowledge gaps and research priorities for future studies to better assess its impacts on the food webs of invaded ecosystems.

Key words: Bloody-red shrimp, exotic species, freshwaters, invasion dynamics, non-native

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Introduction

Freshwater ecosystems are amongst the most affected environments by invasive* species globally (IPBES 2023), resulting in significant ecological, economic and potentially public health impacts, as well as conservation challenges (Diagne et al. 2021; Gippet et al. 2023). Invasive species contribute to biodiversity loss, with freshwater ecosystems experiencing the greatest declines (Reid et al. 2019). These species can drastically alter ecosystem structure and function, especially under the pressures of climate change, which is increasing both the frequency of

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* We acknowledge the current evolution of the definition of the term 'invasive' in the field of ecology. For the purpose of this paper, the term invasive is used to characterise a species that causes ecological or economic harm in a new environment where it is not native.

climate-related disturbances and temperatures favourable to invasions. Understanding invasion processes and predicting their impacts are essential for assessing risks and developing effective management strategies (Moyle and Marchetti 2006; Occhipinti-Ambrogi 2007; Vilizzi et al. 2021). The successful establishment of a potentially invasive species in a new environment is often linked to its life-history traits (also referred as species invasiveness) and other biological characteristics or again to ecosystem invasibility and ecological opportunity, which together facilitate its acclimatisation and integration into the invaded environment (Shea and Chesson 2002; Daly et al. 2023; Su et al. 2023).

The Ponto-Caspian Region, encompassing the Black Sea, Sea of Azov and Caspian Sea, is a significant source of invasive species, particularly crustaceans, which have proliferated within freshwater ecosystems in both Europe and North America (Soto et al. 2023). Amongst these species, the "bloody-red shrimp", *Hemimysis anomala* (G. O. Sars, 1907), has shown rapid range expansion over recent decades, primarily colonising lentic ecosystems such as lakes, canals and river backwaters (Salemaa and Hietalahti 1993; Bij de Vaate et al. 2002; Lantry et al. 2010; Wittmann et al. 2016). Initially introduced to enhance food availability for fish production in ponds across regions of the former Soviet Union (Arbačiauskas et al. 2010), the species' spread was significantly facilitated by the opening of the Main-Danube Canal in 1992. This event enabled the dispersal of *H. anomala* and other Ponto-Caspian species across Europe, mainly through the discharge of ballast water from shipping vessels (Leuven et al. 2009; Minchin and Boelens 2010). The successful invasion of *H. anomala* can be attributed to its high dispersal capability, fecundity, rapid growth rate and ability to colonise habitats with limited trophic resources, whether natural or anthropogenic (Dobrzycka-Krahel et al. 2023; Oliveira et al. 2023). Furthermore, its broad tolerance to variations in temperature and salinity has bolstered its invasive success (Soto et al. 2023). The introduction of Mysidae into lake ecosystems has a well-documented history of unintended ecological consequences. For instance, from the 1950s to the 1980s, *Mysis diluviana* was deliberately introduced into North American lakes and reservoirs to enhance fisheries, yet these introductions frequently resulted in trophic disruptions rather than benefits (Fredrickson 2017). As an efficient zooplanktivore, *M. diluviana* outcompeted juvenile planktivorous fish, altering trophic cascades that restructured entire food webs. In Flathead Lake (Montana), its introduction drastically reduced zooplankton biomass, leading to the collapse of native salmonid populations (*Oncorhynchus nerka*) and subsequent declines in higher predators (Ellis et al. 2011; Devlin et al. 2017). While *H. anomala* shares some ecological traits with *M. diluviana*, it thrives in warmer temperatures, favours littoral and nearshore environments and is an opportunistic omnivore rather than primarily zooplanktivorous (Kipp and Ricciardi 2007; Walsh et al. 2012). These situations underscore the profound and often unpredictable ecological impacts of invasive mysids, emphasising the need for a cautious assessment of *H. anomala*, whose long-term effects remain unexplored and consequently insufficiently understood. Recent observations indicate that the invasion of *H. anomala* is not a localised phenomenon, but a rapidly evolving issue with significant implications for aquatic ecosystems. In Europe, the species has been recorded in a variety of waterbodies, exhibiting an alarming trend in its spread. Notably, recent sightings have expanded to new areas, including lakes in France and Germany and large freshwater reservoirs in the United Kingdom (Frossard and Fontvieille 2018; Andrews et al. 2023; Dickey et al. 2024). In North

America, *H. anomala* was first reported in the Laurentian Great Lakes in 2006 and has since been detected in all five Great Lakes, with the latest discovery in Lake Superior (Marty et al. 2010; Evans et al. 2018). Its recent presence in Lake Michigan harbours further confirms its establishment nearly a decade post-discovery. The rapid establishment of *H. anomala* across various hydrological systems and habitats underscores the urgent need for global monitoring and management strategies to mitigate its impact on local ecosystems.

This study provides a comprehensive review of the current knowledge on the ecology of *H. anomala*. Our investigation includes novel data on the species' habitat preferences, collected through a participatory research approach involving both scientific and French recreational divers. This collaboration facilitated data collection across diverse aquatic environments and promoted community engagement in ecological research, enhancing the monitoring of *H. anomala* populations. Additionally, we assess various methodologies for studying this invasive species, including field observations and sampling techniques. This comparative analysis identifies the most effective methods for detecting *H. anomala* and documenting its spread. By characterising spatial and temporal variations in its occurrence and abundance, we highlight key environmental and biotic factors influencing its further spread. Finally, we identify knowledge gaps and research priorities essential for future investigations into the impacts of *H. anomala* on the food webs of invaded ecosystems.

Material and methods

To support our study of *H. anomala*, a comprehensive literature review was conducted to establish a baseline on the ecological characteristics of the species and gather relevant pieces of information from newly-invaded sites. Initially, the literature review was performed using the Web of Science (WoS, Clarivate Analytics, Philadelphia, USA) databases up to spring 2024, with an update in autumn 2024. A search in Scopus did not yield any additional articles. The literature search employed the following keyword strings: "*Hemimysis*" and "*anomala*" or "Blood* Red" and "Shrimp" or "Blood* red" and "Mysid*" for the title, abstract and keywords. These keywords were selected to capture a broad range of publications, from studies focused specifically on the species to more general discussions. The results from this search underwent exhaustive analysis, encompassing articles published from May 1993 to October 2024 (Suppl. material 1: fig. S1). Additionally, a thorough review of the references and citations of the initially selected papers identified 31 additional articles that provided relevant insights not directly accessible through database searches.

Results and discussion

Mysids belong to the order Mysida, comprising approximately two families, 179 genera and 1,210 species, distributed across a wide range of aquatic ecosystems and habitats (Oliveira et al. 2023). Within this diverse group, the majority of species inhabit marine environments, with only about twenty species found in freshwater and brackish habitats (Mauchline 1980). According to Lunina et al. (2019), the genus *Hemimysis* (family Mysidae) includes 11 species found in marine, brackish and freshwater environments, predominantly inhabiting caves, bottom and demersal regions. Amongst these species is *Hemimysis anomala* (Suppl. material 1: fig. S2), first described by Sars (1907).

Spatial distribution

Native range

Hemimysis anomala originates from the estuaries of the Black Sea, Sea of Azov and Caspian Sea (Fig. 1). It is present at the base of rivers flowing into these seas and in brackish waters. Since the 2000s, *H. anomala* has been reported worldwide, following intentional or accidental introductions (Audzijonyte et al. 2008). Paradoxically, *H. anomala* is recognised as critically endangered in its native habitat (Alexandrov 1999; FAO 2005). This situation illustrates how specific environmental pressures and habitat changes can threaten a species in one context while being considered invasive in another, thereby creating a conservation paradox (Marchetti and Engstrom 2016). In its native range, *H. anomala* faces severe threats from habitat loss, pollution and ecological disturbances (Korzhov 2021). These disturbances, amplified by industrial and agricultural activities (e.g. hydrocarbons, agricultural runoff), alter essential conditions for its survival, impacting water quality and diminishing natural resources and habitats needed by native crustaceans (Zaitsev et al. 2002; Gogaladze et al. 2021; Korzhov 2021). Furthermore, invasive species and ecological shifts heighten competition for resources, further compromising the viability of native populations (Son et al. 2020; Gogaladze et al. 2021).

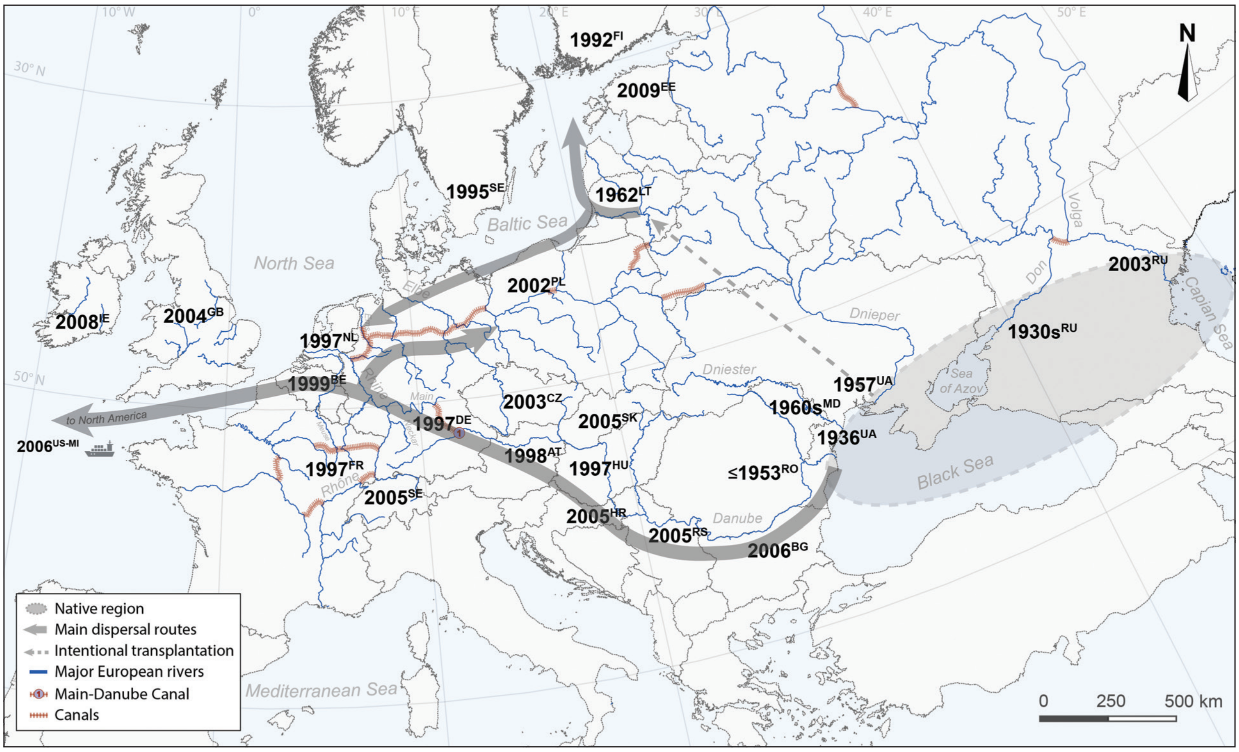

Figure 1. Map showing the distribution and dispersal periods of *Hemimysis anomala* in Central and Western Europe from the Ponto-Caspian area (Black, Azov and Caspian Seas). The shaded area refers to the presumed native range of *H. anomala* (Ponto-Caspian). Wide grey arrows depict the main dispersal routes and dashed grey arrows indicate intentional transplantation to the Kaunas water reservoir in Lithuania (adapted from Audzijonyte et al. (2008)). Solid blue lines represent major European rivers capable of supporting the dispersal of *H. anomala*, while dashed red lines represent canals that have facilitated its spread. The ship pictogram indicates transatlantic transport via ballast water discharge to North America, with the first records of *H. anomala* in Lake Michigan in 2006, likely resulting from multiple European introduction sources (Audzijonyte et al. 2008). Years indicate first records followed by letters indicate the ISO codes of the corresponding country (see Suppl. material 1: table S1).

This duality highlights the complex management requirements for *H. anomala*, where its conservation is critical in its native habitat, but it poses a significant ecological challenge as an invasive species elsewhere.

Spread in Europe

In Europe, the expansion of *H. anomala* and its rate of spread through freshwater ecosystems have been facilitated by human activities since the 1940s (Wittmann 2007). The species was intentionally introduced into large lakes and reservoirs within the former Soviet Union, beginning with the Dniepr Reservoir in Ukraine in the late 1950s and followed by the Kaunas Reservoir in Lithuania in 1960, to serve as fish food (Horecký et al. 2005; Arbačiauskas et al. 2010). These introductions were part of a broader acclimatisation effort conducted between 1955 and 1989 to increase the biomass of commercially important fish species (Karpevich 1975). From the Kaunas Reservoir, *H. anomala* began its expansion into the Baltic Basin via natural dispersal and anthropogenic corridors, including the Curonian Lagoon and connected waterways (Audzijonyte et al. 2008; Arbačiauskas et al. 2017). Molecular analyses confirmed that the Lithuanian populations played a key role as a secondary source for the species' expansion into the Baltic Sea (Audzijonyte et al. 2008). Its ability to tolerate brackish environments (Table 1) facilitated its establishment in the Baltic Sea, where the first observation was recorded in 1992 in the coastal waters of south-western Finland (Salemaa and Hietalahti 1993). Subsequent sightings occurred along the Swedish coastline in 1995, in the Gulf of Gdańsk in Poland in 2002 (Janas and Wysocki 2005) and in Estonian waters in 2009 (Kotta and Kotta 2010). The rapid rate of expansion, confirmed retrospectively in Hungary in 1997, observed in 1998 in Austria and later in 2005 in Slovakia, can be attributed to the release of ballast water from shipping vessels (Wittmann et al. 1999; Wittmann 2007; Borza 2008; Borza et al. 2011). During the early 1990s, the intensification of river traffic across Eastern and Central Europe facilitated the spread of *H. anomala* via the Danube-Black Sea Canal, which connects the Danube River to the Black Sea and its tributaries (Kotta and Kotta 2010). The spread was further enabled by the construction of numerous canals, notably the Main-Danube Canal, which was completed in 1992 and links the Danube to the Rhine Rivers and allows the passage of large vessels from the Black

Table 1. Hydrological and physico-chemical amplitudes of *Hemimysis anomala* (Băcescu 1940; Ketelaars et al. 1999; Wittmann 2007; Ellis and MacIsaac 2009; Wittmann and Ariani 2009), including mean and standard deviation (m ± S.D) for specific parameters (data from Wittmann 2007).

Parameter	m ± S.D	Value (min.-max.)
Depth (m)	4.04 ± 5.42	< 1–60
Temperature (°C)	17.21 ± 4.47	2–28
Dissolved oxygen (mgO_2/l)	7.197 ± 1.42	3.99–13.92
Carbonate hardness (°d)	8.642 ± 0.98	6–12
Conductivity (µS/cm)	3792 ± 6947	98–29200
Water pH (pH)	7.87 ± 0.50	6.21–8.65
Salinity (‰)	2.14 ± 4.21	0–18
Water Current (m/s)	0.15 ± 0.22	0–0.81
Turbidity (NTU †)	28.61 ± 26.29	5–137

Note: † Nephelometric Turbidity Units.

Sea to the North Sea (Fig. 1). The pathway, often referred to as the "southern corridor", further facilitated the spread of the species (Audzijonyte et al. 2008). Beyond continental Europe, the first reports were from the English Midlands in the United Kingdom in 2004 (Holdich et al. 2006), followed by sightings in southeast England in 2020 (Andrews et al. 2023). In Ireland, the first reports date back to 2008, in the basin of the River Shannon, from which the species spread to Northern Ireland (Minchin and Holmes 2008; Minchin and Boelens 2010; Gallagher et al. 2015). Unlike in North America, *H. anomala* has mainly colonised watercourses rather than lake ecosystems in Europe (Verslycke et al. 2000; Janas and Wysocki 2005). The first observation of the crustacean in the Rhine Basin (in Germany and the Netherlands) was made in the Neckar River in 1997 and in the Main River in 1998 (Schleuter and Schleuter 1998; Schleuter et al. 1998; Kelleher et al. 1999). It then continued to spread rapidly in the waterways of north-eastern and southern Germany, as well as in the River Meuse in southern Belgium, with sightings in the Galgenweel, near the Westerschelde Estuary and in the Netherlands (Eggers et al. 1999; Verslycke et al. 2000; Vanden Bossche 2002; Rudolph and Zettler 2003; Stich et al. 2009). The southern corridor has been *H. anomala*'s most likely route into France, where the first observations were made in the Rhône in 2003 (Daufresne et al. 2007) and in the Rhine in 2005 (Dumont 2006). *Hemimysis anomala* may have now established throughout the Paris region and northern France, extending as far as the Rhône Delta in the Mediterranean (Wittmann and Ariani 2009; Dumont and Muller 2010). In the large and deep peri-alpine lakes of France, while the species has colonised Lake Bourget and Lake Geneva since 2007 (Golaz and Vainola 2013; Frossard and Fontvieille 2018; Lods-Crozet 2020), the first individuals were only recently discovered in Lake Annecy in early 2024, likely due to this system being disconnected from the Rhône River (SILA, pers. com. 2024). The identification of *H. anomala* in Lake Stechlin in Germany in 2023 now raises concerns about its spread and potential impact on local biodiversity (Dickey et al. 2024).

Spread in North America

Hemimysis anomala was first observed in Lake Ontario in the Great Lakes region of North America in 2006 (Ricciardi 2006; Kipp and Ricciardi 2007; Marty et al. 2010), prompting the establishment of a monitoring network to document and assess its expansion across the continent (Suppl. material 1: fig. S3). The species has since colonised other large lakes (Michigan, Muskegon) via their connecting rivers (Pothoven et al. 2007), as well as the St. Lawrence River near Montreal, Canada (Kestrup and Ricciardi 2008; de Lafontaine et al. 2012). Similar to Europe, the arrival and further invasion of *H. anomala* were facilitated by ballast water exchanges from multiple European source regions. Genetic analyses confirmed that North American populations originated from both Danubian and Baltic lineages (Audzijonyte et al. 2008; Questel et al. 2012), suggesting repeated introductions through ballast water releases. By 2018, it had been found in all five Laurentian Great Lakes (Suppl. material 1: fig. S3; Evans et al. (2018)).

Introduction vectors in isolated environments

Although the spread of *H. anomala* has been mainly facilitated by ballast water, other introduction vectors must be considered in isolated environments like Lake Annecy. Amongst these vectors, recreational activities such as boating, diving and fishing can play a significant role (Martínez-Laiz et al. 2019; Morreale et al. 2023). The passive transport of aquatic organisms via contaminated watercraft and biofouling on boat hulls, especially in the absence of strict cleaning protocols, provides an effective mode of dispersal (Kelly et al. 2013; Mohit et al. 2023). Furthermore, fish stocking and the transport of live fish represent an important anthropogenic vector, as the water used in transport can harbour larvae or juveniles of *H. anomala* (Zajicek et al. 2009; Saccà 2015; Olden et al. 2021). Strengthening regulations on fish transport and ensuring water treatment or filtration before discharge could help mitigate this risk. Lastly, although natural vectors are less studied, extreme weather events such as flooding and passive dispersal by aquatic birds (especially migratory species) could serve as secondary mechanisms of transport, leading to the accidental introduction of these organisms into new habitats (Andrews et al. 2023). These factors highlight the need for comprehensive management strategies to mitigate the risks of *H. anomala* introductions.

Comparative analysis of sampling methods and advanced analytical approaches

Most studies conducted between the early 1990s and 2010 focused on describing the expansion and ecology of *Hemimysis anomala* (Salemaa and Hietalahti 1993; Verslycke et al. 2000). In the large North American lakes, study sites were selected, based on the preferred habitats of *H. anomala*, with individuals often collected by deploying a plankton net from the surface (Marty et al. 2010; Taraborelli et al. 2012). In other colonised habitats such as rivers, canals and artificial shelters, various methods have been used or developed to detect, sample and quantify their abundance, including direct observations at night using torchlight (Holdich et al. 2006; Stubbington et al. 2008), light-based traps (Brown et al. 2017), baited traps (Odenwald et al. 2005), hand nets (Faasse 1998; Janas and Wysocki 2005; Pothoven et al. 2007), dredging (Kotta and Kotta 2010), direct observation and sampling using a home-made collector by diving (Dumont 2006; Dumont and Muller 2010; Jacquet 2023; Frossard et al. 2023), environmental DNA (eDNA) (Oyagi et al. 2017) and, more recently, high-resolution acoustic cameras (multibeam sonar; Rogissart et al. (2024)).

The use of light, such as torchlight, can be highly effective in detecting the presence or absence of *H. anomala* in shallow waters, although turbidity can restrict this approach (Stubbington et al. 2008). Sampling using baited bottles containing algae tablets (*Spirulina* 20%) intended for aquarium fish has also proved effective overnight at shallow depths (Odenwald et al. 2005). However, using pieces of fresh pig's liver can lead to significant oxygen consumption in baited bottles, resulting in increased mortality (Odenwald et al. 2005). Although diving-based methods necessitate specific skills and, depending on the country, recognised certification, they enable direct observation, sampling and *in situ* experiments, although they remain rarely used in long-term studies (Dumont and Muller 2010; Frossard and Fontvieille 2018; Jacquet 2023). Diving can improve understanding of certain aspects of the animal's life, such as the proportion of females (e.g. with visible marsupial pouches) or juveniles or the seasonality of the appearance of swarms in monitored areas.

Although vertical nets and traps have been effective at night for assessing Mysidae populations (Brooking et al. 2010; Brown et al. 2017), more advanced methods have also emerged in recent years. Efforts have been made to use metabarcoding to detect *H. anomala* in Great Lake ecosystems or in Lake Geneva by analysing the stomach contents of predatory fish, although its presence was not detected (Mychek-Londer et al. 2020; Rogissart et al. 2022).

Environmental DNA (eDNA) sampling, particularly when combined with quantitative PCR (qPCR) or droplet digital PCR (ddPCR), provides a highly sensitive, non-invasive method for detecting *H. anomala* in challenging environments such as ballast water and complex habitats, often missed by traditional methods (Oyagi et al. 2017; Cangelosi et al. 2024; Melliti et al. 2025). While eDNA-qPCR has great potential for early detection, environmental factors such as water flow, temperature and turbidity can affect detection rates and lead to false positives or negatives, as eDNA cannot distinguish between living and dead organisms (Oyagi et al. 2017; Cangelosi et al. 2024). Recent advances in ddPCR technology provide increased sensitivity for detecting low concentrations of eDNA and remove the need for calibration curves (Melliti et al. 2025). Therefore, eDNA serves as a valuable complementary tool to traditional methods in monitoring programmes. Additionally, Rogissart et al. (2024) demonstrated that using an acoustic camera can be a valuable non-invasive approach for monitoring and recording real-time behaviour of *H. anomala* in lake littoral zones.

Overall, integrating traditional and advanced sampling techniques enhances our understanding of *H. anomala*'s ecological impact on invaded ecosystems and underscores the importance of continued innovation in methods to more effectively monitor invasive species.

Habitat

Habitats with rocky substrates, such as those found in large lakes or ponds and built environments like concrete walls with cracks, provide favourable conditions for the establishment of this species (Kestrup and Ricciardi 2008; Marty et al. 2010; Walsh et al. 2010; Boscarino et al. 2020). Additionally, the arrangement of rocks, their diameter and the distance between them or between them and the benthic sediments influence the speed of colonisation by providing suitable habitats and substrates to facilitate their establishment and growth (Claramunt et al. 2012). Swarm areas are also larger in inland lakes than in coastal rivers (Kestrup and Ricciardi 2008). Observations of habitat preferences depend on the sampling methods: while the average swarm depth is around 30 m in North American studies, it exceeds 60 m in the initial studies conducted in the Ponto-Caspian Region and around the Black Sea (Zhuravel 1959). However, in general, *H. anomala* are thought to prefer sublittoral waters (< 40 m) (Ricciardi et al. 2012). Numerous swarms have been observed at depths < 12 m within and around beds of submerged branches or macrophyte *Myriophyllum spicatum* (Serge Dumont, pers. com.) and anthropogenic structures such as under pontoons, riprap, jetties, slipways and in tree roots along the banks (Stubbington et al. 2008). *Hemimysis anomala* primarily lives hidden, near or under benthic or artificial shelter and rocky substrates during the day, where it finds refuge from strong currents or predators (Ketelaars et al. 1999; Rogissart et al. 2024).

Diet and feeding behaviour

The diet of *H. anomala* was initially identified through analyses of stomach content analyses, stable isotopes and laboratory-based feeding experiments. Stable isotope analyses of stomach contents and microscopic examination of both *H. anomala* and its predators, along with DNA marker analyses specific for hard-to-analyse prey species in gut contents, provide valuable insights into the trophic position of *H. anomala* (Evans et al. 2018; Frossard et al. 2023). Immunochemical gut content analysis using specific antisera complements these methods by enabling precise identification of prey species, thereby providing a more detailed and comprehensive understanding of the feeding ecology of *H. anomala* (Wachala et al. 2025). When coupled with regular seasonal sampling, these methods offer a clear picture of the trophic ecology and their role in native food web interactions (Lantry et al. 2012; Marty et al. 2012; Yuille et al. 2012; Evans et al. 2018; Frossard et al. 2023; Patonai et al. 2024).

Juveniles generally consume small zooplankton such as rotifers (Halpin et al. 2013) and phytoplankton (Ketelaars et al. 1999; Borcherding et al. 2006; Frossard and Fontvieille 2018). Phytoplankton can also be an important resource for all life stages, as observed in Lake Ontario (Evans et al. 2018). Adults primarily consume zooplankton, but, being cannibalistic, are also capable of consuming various organic remains (dead fish) (Ketelaars et al. 1999; Dumont 2006) and ingesting green algae (Lowery et al. 2023). Their maximum capacity to ingest algae is approximately 1,000 cells per animal per hour and 9,000 cells per ml at half-saturation (Lowery et al. 2023). Field observations suggest that *H. anomala* prefers cladocerans, regardless of their life stage (Halpin et al. 2013; Evans et al. 2018). These findings are supported by laboratory experiments in which *H. anomala* preferentially entered mesocosms with a majority of cladocerans as resources (Iacarella et al. 2015). *Hemimysis anomala* can consume prey up to 30% of its size, including *Bythotrephes longimanus* that is a crustacean predator (Evans et al. 2018). Its primary filter (structure enabling the filtration of food particles), with a mesh size of less than 1 µm, comparable to that of *Daphnia* spp., enables it to exploit a wide range of suspended particle sizes, potentially granting access to an abundant food source (Borza et al. 2024). Additional immunochemical analyses confirm its broad dietary range, which includes a wide variety of prey, including *Bosmina longirostris*, *Daphnia pulex*, veliger larvae of Dreissena mussels and copepods (Wachala et al. 2025).

Hemimysis anomala shows marked seasonal variations in its diet, closely linked to fluctuations in prey abundance, ranging from autotrophic protists to zooplankton (Evans et al. 2018; Frossard et al. 2023). This dietary flexibility enables *H. anomala* to adjust its feeding strategy according to seasonal and environmental variations, maximising its success in various invaded ecosystems (Patonai et al. 2024). The species is likely to utilise greater food abundance in spring and summer than in autumn and winter, showing a predominant dependence on the pelagic environment during summer and autumn (Frossard et al. 2023). Additionally, higher temperatures from spring to autumn tend to increase feeding rates in parallel with increasing metabolic demand, enhancing growth potential (Sun et al. 2013; Penk et al. 2016). *Hemimysis anomala* is often found close to the substrate, with a significant proportion of detritus in its stomach content, suggesting direct feeding on benthic substrates (Borcherding et al. 2006). However, the relative contribution of benthic and pelagic resources appears to vary depending on environmental characteristics and opportunities at study sites, demonstrating its significant dietary flexibility (Ives et al. 2013; Frossard and Fontvieille 2018; Patonai et al. 2024).

Spatio-temporal distribution and behaviour dynamics

The distribution and behaviour of *H. anomala* swarms exhibit significant spatio-temporal dynamics. The depth of plankton net catches according to the time of day or night during fishing revealed vertical variations in swarms during the day, even in a marina with a maximum depth of around 2 m (Nunn and Cowx 2012). Catches appear to be much deeper and closer to the substrate during the day than at night. This circadian rhythm variation can be explained by the influence of light intensity and spectrum on the distribution and behaviour of swarms (Boscarino et al. 2012). Experiments exposing adults and juveniles to different light spectra of increasing intensity revealed that juveniles preferred brighter light sources than adults (10^{-3} and 10^{-7} mylux, respectively; 'mylux' measures brightness as perceived by the visual pigments of *H. anomala*, Gal et al. (1999)), with adults preferring to remain hidden during the day ($> 10^{-4}$ mylux for avoidance; Boscarino et al. (2020); Rogissart et al. (2024)).

Seasonal variations in swarm abundance and density have also been observed. Observations in Germany over one year (2005) revealed variations in abundance across different periods (Janas and Wysocki 2005). In summer, the proportion of adults was lower than juveniles and vice versa in winter. This dynamic is also observed in North America, where summer populations of *H. anomala* are predominantly composed of juveniles (< 6 mm), small adult males (~ 7 mm) and few reproductive females (5–10%). In winter, *H. anomala* forms swarms that can be observed in both Europe and North America, with densities peaking when temperatures are between 6 °C and 12 °C (Verslycke et al. 2000; Claramunt et al. 2012; Jacquet 2023; Rogissart et al. 2024). The size composition or sex/developmental stage of individuals present in these swarms do not differ from those of individuals sampled on the bottom, indicating that these aggregations are not specifically related to reproduction function (Wachala et al. 2025). This pattern can be explained by temperature influences, food availability and predator presence, as confirmed by both laboratory and field studies (Boscarino et al. 2020; Wachala et al. 2025). *Hemimysis anomala* can adapt to a relatively wide range of abiotic factors and environmental conditions, including low winter temperatures (Table 1; Pienimäki and Leppäkoski (2004)). Individuals exhibited sustained growth even during winter at the lowest temperatures (0.011–0.015 mm d^{-1}) and, contrary to previous assumptions, post-reproductive males do not die immediately after reproducing (Wachala et al. 2025). This ability to survive in cold waters aligns with observations from frozen (surface) lakes in France, where temperatures reach approximately 3 °C (Dumont 2006), suggesting high thermal tolerance and adaptation to prolonged winter conditions.

Once introduced into a habitat, *H. anomala* can spread in lentic environments, but is displaced by flowing water (Table 1), limiting its introduction into new ecosystems (Wittmann and Ariani 2009). However, the species has spread across England and the Baltic Region, demonstrating some ability to migrate upstream through unsuitable areas for permanent inhabitation (Stubbington et al. 2008). *Hemimysis anomala* may have a higher capacity to spread compared to other invasive Ponto-Caspian crustaceans, such as certain gammarids established in Europe and North America (Pienimäki and Leppäkoski 2004; Wittmann 2007).

Ecological impacts

Potential negative impacts

The ecological impacts of *Hemimysis anomala* establishment in receiving aquatic ecosystems are predominantly based on its high zooplankton consumption capacity (Borcherding et al. 2006; Ricciardi et al. 2012; Lowery et al. 2023). For instance, in the St. Lawrence River, densities of cladocerans, ostracods, rotifers and predatory invertebrates decreased drastically in late summer, corresponding with the proliferation of Mysidae (Sinclair et al. 2016). Similarly, the abundance of cladocerans declined sharply following the invasion of Lake Honderd in the Netherlands by *H. anomala* (Ketelaars et al. 1999). This predation pressure can disrupt zooplankton community structure, potentially altering food web dynamics and impacting species dependent on zooplankton as a primary food source (Fig. 2).

In addition to predation, *H. anomala* may compete directly with native Mysidae for zooplankton, a competition intensified by *H. anomala*'s higher feeding rates relative to body mass compared to other Mysidae (Dick et al. 2013; Patonai et al. 2024). This competitive advantage is highlighted by the stronger functional response of *H. anomala*, where its higher attack rates and shorter handling times enable efficient predation, even in the presence of predators, distinguishing it from native Mysidae (Barrios-O'Neill et al. 2014; Penk et al. 2018). The species may also compete with other native macroinvertebrates with which it shares a trophic niche. However, *H. anomala*'s impact on such macroinvertebrates (e.g. gammarids) does not appear significant (Marty et al. 2010; Taraborelli et al. 2012). Further, in the gravel pits of Alsace (France), no significant impact of *H. anomala* on zooplankton resources or Hydra populations has been demonstrated, despite both species feeding on zooplankton and are present at the same depths (Dumont and Muller 2010).

Beyond trophic interactions, *H. anomala* exhibits the ability to ingest 30 µm plastic particles at a similar rate to microalgae, with a maximum ingestion of about 750 particles per animal per hour (half-saturated at 5,000 particles per ml) (Lowery et al. 2023). Furthermore, *H. anomala* was associated with high contaminant concentrations, including methylmercury (MeHg), exceeding that of other littoral invertebrates such as amphipods, dreissenid mussels and zooplankton (Zhang et al. 2012; Brown et al. 2022). This capacity raises concerns about the bioaccumulation of contaminants up the food chain, potentially impacting fish and other higher trophic levels. Due to its position in invaded food webs and its prevalence in some places, such as harbours, for instance, where high levels of organic and inorganic pollutants can be detected, *H. anomala* may contribute to the bioaccumulation of these pollutants. Furthermore, its presence can potentially elongate the food web by introducing additional trophic levels, thereby increasing the risk and magnitude of contaminant transfer to higher trophic levels. Additionally, its high lipid content not only facilitates the retention of organic contaminants, but also provides an energy-rich resource for consumers, raising questions about whether the overall impacts of *H. anomala* invasion on food webs are beneficial or detrimental.

Potential positive impacts

Despite the potential for negative impacts, *H. anomala* may contribute positively to certain ecosystems, especially as a food source for various fish species. Isotopic approaches and visual examination of stomach contents suggest that *H. anomala* can contribute to the diet of various fish species such as yellow perch (*Perca flavescens*), European perch (*Perca fluviatilis*), rock bass (*Ambloplites rupestris*), lake cisco (*Coregonus artedi*), white perch (*Morone americana*), alewife (*Alosa pseudoharengus*), largemouth bass (*Micropterus salmoides*) and rainbow smelt (*Osmerus mordax*) (Lantry et al. 2010; Yuille et al. 2012; Gallagher et al. 2015; Geisthardt et al. 2022). The contribution of *H. anomala* to fish diets appears positively correlated with its abundance and varied

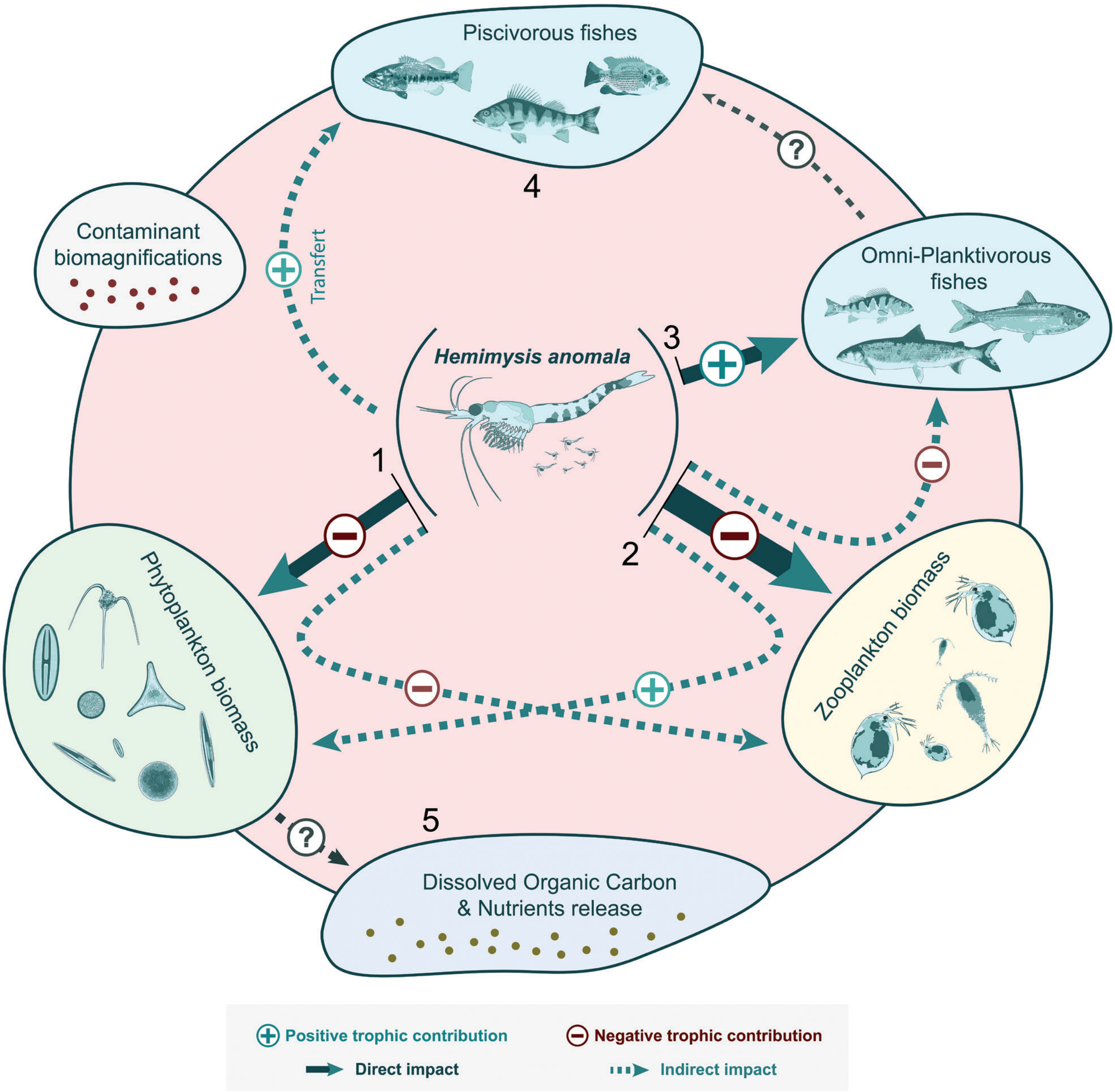

Figure 2. Impacts of *H. anomala* in aquatic food webs. *Hemimysis anomala* influences food web dynamics through multiple direct and indirect pathways leading interferences with different trophic levels. Solid arrows represent direct trophic interactions, while dashed arrows shown indirect effects. (1) *Hemimysis anomala* exerts a direct negative impact on phytoplankton through consumption, reducing its biomass that can trigger indirect negative impacts on zooplankton by lowering the availability resources; (2) *Hemimysis anomala* exerting a direct negative impact on zooplankton biomass by predation that can lead to a positive indirect effect on phytoplankton biomass by reducing grazing pressure from zooplankton. However, the decline in zooplankton may have negative indirect consequences for omni-planktivorous fishes, which rely on zooplankton as a primary food source; (3) *Hemimysis anomala* serves as a prey for omni-planktivorous fishes, potentially increasing their biomass (direct positive effect). However, by reducing zooplankton availability, *H. anomala* may impose an indirect negative effect on these fishes due to resource competition. As a consequence, the net effects of *H. anomala* on omni-planktivorous fishes remain to be clarified; (4) Due to the uncertainty of the lack of effect of *H. anomala* on omni-planktivorous fishes, the indirect effects of *H. anomala* on piscivorous fishes remain unclear. However, *H. anomala* may contribute to contaminant biomagnification by lengthening the food web ultimately impacting the extent of contamination of higher trophic levels; (5) *Hemimysis anomala* releases nutrients and dissolved organic carbon (DOC) through excretion and partial fragmentation of organic matter during feeding, which may stimulate primary production. This process may play a role in nutrient cycling and biogeochemical processes in aquatic ecosystems, though further research is also needed here to quantify its extent.

amongst seasons and years. However, it is important to note that, despite the presence of swarms of *Hemimysis* at the same sites, they are found in low quantities or even absent in the stomach contents of the round goby (*Neogobius melanostomus*) (Lantry et al. 2010; Fitzsimons et al. 2012; Geisthardt et al. 2022). Moreover, the high lipid concentration of these shrimps enables young perch to reach sexual maturity much more quickly than with a diet based on other prey (Zhuravel 1959; Borcherding et al. 2007). These observations suggest that *H. anomala* could have a significant impact on food web dynamics and the feeding ecology of fish in environments favourable to the species where it occurs at high abundance (Fig. 2; Geisthardt et al. (2022)).

Finally, *H. anomala*'s omnivorous feeding habits, combined with its ability to actively swim, enable it to exploit both benthic and pelagic habitats. The ecological versatility may mitigate some ecological disruptions caused by other invasive species and also participate to food-web stability through energetic coupling (Rooney et al. 2006; McMeans et al. 2016). This dual habitat exploitation allows *H. anomala* to adapt to varying environmental conditions and seasonal shifts in resource availability, which likely explains part of its invasive success. For example, its nycthemeral movements into pelagic waters could reduce the negative ecological impact of the energy sink induced by zebra or quagga mussels (*Dreissena polymorpha* and *D. bugensis*) (Yuille et al. 2012; Ives et al. 2013). Some of the nutrients captured by these mussels and released as faeces may be assimilated by *H. anomala* and made available again to consumers such as fish, particularly in areas with high densities of zebra mussels, such as the littoral zones of large lakes (Brown et al. 2022). The ability to reconnect benthic and pelagic food webs particularly relevant in the Laurentian Great Lakes where the process of benthification has been reported (Hecky et al. 2004).

Indirect interactions with native and invaded species

Beyond direct ecological impacts (i.e. predation and competition), *H. anomala* may exert indirect influences on native and invaded communities by altering trophic interactions, habitat use and ecosystem processes. Recurrent *in situ* and direct observations suggest a potential interaction between *H. anomala* and the signal crayfish (*Pacifastacus leniusculus*), with the possibility that the crayfish may provide a kind of refuge against perch predation (Jacquet 2023). While this interaction has yet to be confirmed, this association may facilitate the persistence and local proliferation of *H. anomala* populations in benthic habitats, especially during periods of high predation pressure. In habitats shared with native benthic species, *H. anomala* may indirectly impact these organisms by competing for limited shelter or changing habitat and food availability (Marty et al. 2010; Penk et al. 2018). The presence of *H. anomala* may not only affect species distributions, but may significantly influence broader ecosystem dynamics, including sediment bioturbation and nutrient cycling (Covich et al. 1999; Ricciardi et al. 2012). For example, crustaceans like *Mysis relicta* have been shown to influence oxygen fluxes and sediment biogeochemistry through their bioturbation activity (Lindström and Sandberg-Kilpi 2008). Furthermore, the trophic position of *H. anomala* in aquatic food webs reveals its dual role as a prey item for planktivorous and omnivorous fishes and as a consumer of zooplankton and phytoplankton, potentially disrupting energy flow and nutrient dynamics (Pérez-Fuentetaja and Wuerstle (2014); Fig. 2). By reducing zooplankton grazing pressure, *H. anomala* may indirectly promote phytoplankton blooms, altering nutrient release and dissolved organic carbon (DOC) cycling (Ricciardi et al. 2012). However, direct consumption of phytoplankton by *H. anomala* can exert a negative effect, reducing biomass at the base of the food web (Ketelaars et al. 1999; Marty et al. 2012; Sinclair et al. 2016). As a result, the net effect on nutrient dynamics fluctuates between positive and negative, depending on specific ecological contexts and trophic interactions. Additionally, this alteration of trophic dynamics could affect higher trophic levels, such as piscivorous fishes, through cascading effects of reduced prey availability (Pérez-Fuentetaja and Wuerstle 2014; Brown et al. 2022). These interactions raise questions about the ecological relationships of *H. anomala* with other organisms and its role in structuring invaded and/or endemic communities.

Management and control efforts

Biological monitoring and early detection

Regular monitoring in aquatic ecosystems is essential to detect *H. anomala* during the early stages of establishment, making it possible to monitor the colonisation front of the species to reduce its spread. Advanced monitoring methods, including environmental DNA sampling (eDNA), have proven efficient and rapid for detecting the species (Cangelosi et al. 2024; Melliti et al. 2025). The possibility of leaving light-based traps in place for control purposes (Brown et al. 2017) adds a new dimension to ecological monitoring.

Control solutions

To prevent and effectively manage the spread of invasive species, it is important to disinfect objects frequently in contact with infested waterbodies, such as fishing gear, boats, trailers, sampling and diving equipment and waders. These items can unintentionally transport live specimens or propagules to new ecosystems. Disinfection is necessary because *H. anomala* can survive on damp surfaces or in residual water, enabling its accidental introduction into non-invaded habitats. Several disinfection methods have been developed and tested to evaluate their effectiveness in reducing the spread of this species. Disinfectant-based aquatic treatments (e.g. Virkon™ Aquatic, Virasure™ Aquatic) and the use of steam have demonstrated 100% mortality of *H. anomala* specimens, suggesting here their effectiveness in inhibiting the spread of this invasive species (De Stasio et al. 2019; Coughlan et al. 2020). Treatments show that spraying is less effective than immersion (De Stasio et al. 2019). Additionally, treatment with hot water at 45 °C for 15 minutes resulted in 99% mortality in *H. anomala*, making this protocol a simple, rapid and effective biosecurity method for preventing the spread of this species (Anderson et al. 2015). Finally, drying, although less effective, requires approximately 8 days to achieve significant mortality (Anderson et al. 2015). Exposure to ultraviolet (UV) radiation showed a significant reduction in the survival of *H. anomala*, which has a very low tolerance to UV, reaching a value of 17.8 kJ/m^2 (Zeisler 2023). These disinfection methods, including UV exposure after validation, should be incorporated into biosecurity protocols to decontaminate equipment and prevent the spread and impact of *H. anomala* within aquatic ecosystems.

Public awareness and invasive species policy

Public awareness of invasive species policy plays a significant role in preventing accidental spread, especially in areas with recreational fishing or boating. Invasive species policies addressing education, such as decontamination protocols for boats and equipment, can reduce the risk of spread. For instance, implementing rigorous cleaning protocols for fishing and water sports equipment and boats is essential to mitigate the risk of unintentional introductions and dispersal (Coughlan et al. 2020; Mohit et al. 2023). This has been proposed for some very aggressive aquatic species during the last decade, for instance for quagga mussels (Wong and Gerstenberger 2015).

Conclusions and future horizons

The ecological role and threats of *Hemimysis anomala*

Hemimysis anomala appears to have invaded virtually all types of freshwater ecosystems, including lakes and rivers worldwide, as well as brackish environments such as the Baltic Sea. While this invasion is no longer in doubt, the consequences, along with the ecological and socio-economic risks, remain poorly understood. This underscores the need for effective monitoring and management strategies. Our study demonstrates the ecological role of this Mysidae within food webs, particularly through its interference with native zooplankton and its ability to exploit a wide range of habitats. This plasticity poses a threat to local fauna as it may promote changes in community structure and dynamics.

Key knowledge gaps and research priorities

Further research is necessary to fill the gaps in existing knowledge about *H. anomala*. These studies should include a detailed analysis of its life cycle parameters in the natural environment, such as reproductive patterns, longevity and population dynamics including swarming events. Notably, gut content analyses reveal that swarming may primarily serve as a feeding function rather than reproduction (Wachala et al. 2025). It is also important to investigate its periods and sites of reproduction, along with its interactions with biotic and abiotic factors, specify the effect of temperature on its reproductive cycle, as well as prey-predator interactions and food resources. More detailed studies of migration and homing behaviour, particularly their energetic demands, physiological drivers such as sensory mechanisms and responses to environmental cues, could also provide valuable insights into the ecophysiology of the animal. As recently observed in *H. margalefi*, which relies on the chemical signature of its habitat to navigate circadian migrations (Derrien et al. 2024), *H. anomala* might similarly depend on such mechanisms. Investigating these processes, particularly by identifying and characterising the chemical compounds involved, would improve our understanding of its behavioural ecology. Although *H. anomala* has established populations in several brackish environments, including the Baltic Sea (Salemaa and Hietalahti 1993; Janas and Wysocki 2005; Kotta and Kotta 2010), almost nothing is known about its long-term persistence and ecological interactions in such habitats. The species' ability to tolerate variable salinities (Table 1), its reproductive success and its competitive interactions with native and non-native species in estuaries remain insufficiently documented. Additionally, the role of *H. anomala* in nutrient cycling and dissolved organic carbon (DOC) dynamics remains poorly understood. While this species releases nutrients and DOC through excretion and organic matter decomposition, the extent to which these processes influence primary production and microbial activity remains uncertain. Identifying its impact on nutrient remineralisation, microbial interactions and potential contributions to biogeochemical cycles would refine our understanding of its ecological role in aquatic ecosystems. Finally, its influence on higher trophic levels, particularly piscivorous fishes, remains unclear and requires further investigation. *Hemimysis anomala* interacts with these predators both directly, through biomagnification and contaminant transfer and, indirectly, via contrasting trophic effects (Fig. 2). However, its net impact on piscivorous fish populations is not well established. Further research is needed to determine how these mechanisms vary across ecosystems and influence food web stability.

Towards comprehensive monitoring and management

We believe that expanding our knowledge of the distribution of *H. anomala* is another priority. A multi-scale and multi-methods approach combining diving, eDNA and remotely operated vehicle surveys would enable us to map its distribution across and within ecosystems. To refine our understanding of its place in food webs, this will require the application and development of complementary approaches such as underwater video, metabarcoding and immunochemical analyses.

Leveraging citizen science for invasive species monitoring

The creation of participatory diving networks can mobilise recreational divers to report the presence of *H. anomala*, thereby increasing monitoring efforts in often inaccessible areas. These collaborative initiatives not only collect valuable data, but also raise community awareness of the problem of invasive species.

This study demonstrates the potential of citizen science to enhance invasive species monitoring while promoting public engagement in biodiversity conservation. Through participatory diving networks, significant data were gathered on *H. anomala*'s presence, habitat use and seasonality, showcasing the benefits of collaborative initiatives (Appendix 1). Observations were conducted over an annual cycle, recording *H. anomala* occurrences and habitat types. Divers contributed data using standardised questionnaires, which included four habitat categories (e.g. benthic sediments covered by quagga mussels, crevices under rocky outcrops, wrecks and artificial shelters and the water column). They also estimated relative abundances of the species and provided additional information, such as life stages (adult vs. juvenile).

By combining scientific diving with citizen science, this study elucidated a clear seasonal pattern in the habitats occupied by *H. anomala* (Fig. 3). Winter swarms, often exceeding 160,000 individuals in approximately 5 m^3 and occasionally occupying volumes up to 100 m^3, were widely observed at depths of 10 to 20 metres. In contrast, summer sightings were rare, with individuals sheltering under rocks or at greater depths (approximately 1000 individuals per 5 m^3).

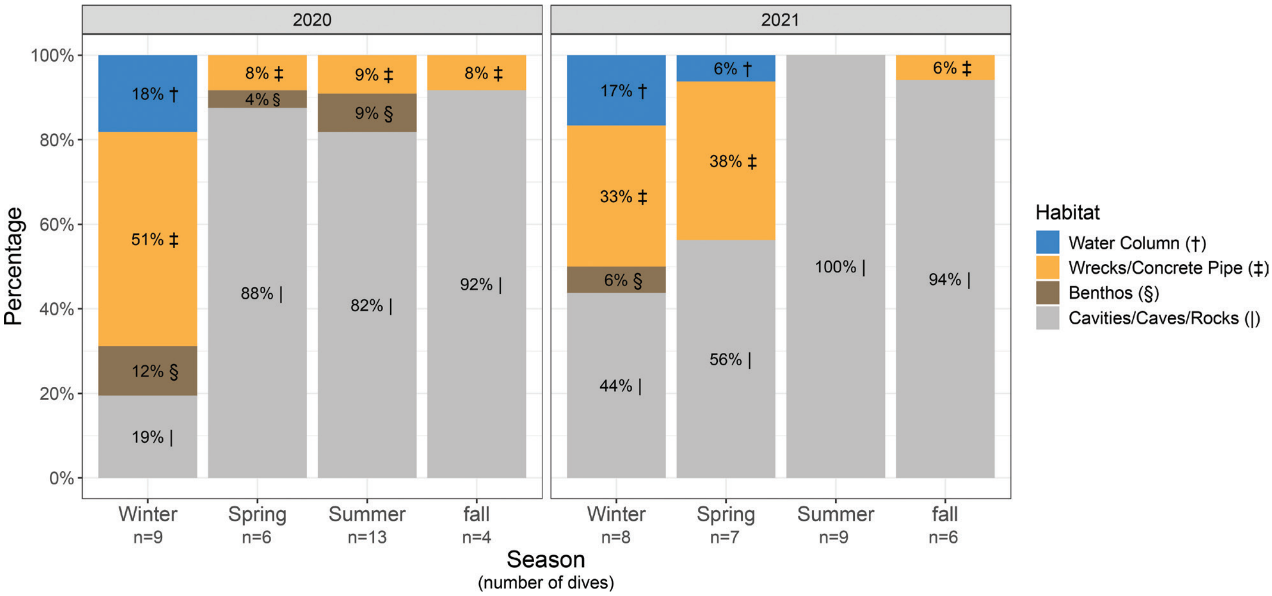

Figure 3. Percentages of habitat types occupied by *H. anomala* observed during the 2020–2021 seasons in Lake Geneva, at the Saint-Disdille pilot site (Thonon-les-Bains, France; Appendix 1). Data were collected through scientific diving and citizen science, with the total number of dives indicated below the bars (n). Observed habitats include the water column (†), anthropogenic structures such as wrecks and concrete pipes (‡), benthic habitats (§) and cavities, caves or rocks (|). The video reveals what winter swarms look like in Lake Geneva (https://hal.science/hal-04820062).

This seasonal pattern underscores the ecological flexibility of *H. anomala* and its adaptation to varying environmental pressures. These results demonstrate the value of citizen science in advancing invasive species monitoring, offering both enhanced data collection capabilities and a means of engaging the public in biodiversity conservation efforts.

Conclusion

Despite promising developments, early detection using new methodologies remains under-explored in current monitoring programmes. These tools need to be further integrated to provide an early warning of its presence and better respond to its expansion. These efforts should also include predictive modelling to assess potential geographic and ecological spread, especially under climate change and habitat alteration scenarios. Additionally, participatory research, such as diving, should be conducted to increase public awareness and cooperation, providing valuable data and encouraging citizen engagement in addressing the spread and impacts of invasive species that threaten the biodiversity of aquatic ecosystems.

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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: SJ. Data curation: HR. Investigation: HR, SJ. Project administration: SJ. Supervision: SJ. Visualization: HR. Writing - original draft: JG, HR. Writing - review and editing: HR, VF, JM, SJ.

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Data availability

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

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Appendix 1

Recreational divers as participative citizen science

This appendix provides additional methodological details about the participatory diving study conducted between 2020 and 2021. The primary site was located in Lake Geneva at Saint-Disdille, Thonon-les-Bains, France (46°24'06"N, 6°30'08"E DMS), which is characterized by diverse habitat types.

Observations were made elsewhere, at eight sites in Lake Geneva, two in Lake Bourget (France), and one in Lake Annecy (France) through 120 scuba diving expeditions (63 scientific and 47 recreational; total immersion duration of 72.5 hours) conducted both during the day and at night over an annual cycle to record *H. anomala* occurrence and habitat type.

Data collection relied on questionnaires completed by divers (see Fig. S4). Divers recorded habitat categories (e.g., benthic sediments primarily covered by quagga mussels, crevices under rocky outcrops, wrecks and artificial shelters such as harbors and other submerged structures, and the water column) and estimated the relative abundance of the species (e.g., < 50, 50–100, 100–500, 500–1000, > 5000 individuals) and identified the life stage of the animals (adult vs. juvenile).

The survey revealed important seasonal variations.

- During winter, numerous dense swarms of *H. anomala* were most frequently observed at diving sites. They are widely seen in open water near wrecks and inside them, occupying significant volumes with millions of individuals. The swarms are mostly observed between 10 and 20 meters deep and consist mainly of adult individuals.
- From spring onwards, *H. anomala* swarms are not observed in the water column and become increasingly rare, likely due to high mortality and strong predation pressure exerted by perch, which ascend from the depths during this time of the year (unpublished data). By May, the swarms disappear, and only a few tens to hundreds of individuals, mostly juveniles, are detected under rocks, in holes, on the ceilings of submerged pipes, and/or at greater depths.
- During summer, individuals are difficult to observe and generally shelter at the bottom of crevices, often associated with crayfish, or under rocks and stones. Once, a dense 1-m^3 swarm of adults was observed at a depth of 45 m at a diving site in Evian (France), and groups of 10–20 individuals were reported in front of crayfish burrows at depths of 42–54 m (2020).
- During early autumn, the mysids were observed in small numbers under rocks, at the bottom of crevices, but regularly with an increasing number of individuals compared to the summer period. During the second part of autumn, swarms begin to reform and return to open water, devoid of apparent predators. However, it is important to note that during the winter of 2022–2023, a significant absence of swarms was observed, which may be linked to unusually high temperatures that impacted their behaviour and population dynamics (Jacquet, 2023).

Supplementary material 1

Additional information

Authors: Hervé Rogissart, Jérôme Marty, Jonathan Grimond, Victor Frossard, Stéphan Jacquet

Data type: docx

Explanation note: This file includes one table and four supplementary figures.

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Research Article

Invasive signal crayfish and native noble crayfish show trophic niche shrinkage in sympatry

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Abstract

Studying the coexistence of native species and invasive species with similar functional traits, habitat usage, and feeding habits is crucial for understanding the dynamics of invasion and ecological changes in the invaded ecosystem. Due to competitive exclusion and often also dissemination of crayfish plague pathogen (*Aphanomyces astaci*, Schikora), North American crayfish represent a major threat to European native crayfish. Their co-occurrence is often only temporary, making studies investigating trophic ecology of native and non-native crayfish species rare. In this study, trophic niche and feeding ecology of European native noble crayfish *Astacus astacus* (Linnaeus, 1758) and North American signal crayfish *Pacifastacus leniusculus* (Dana, 1852) were compared between their sympatric and allopatric sites, in Křesánovský brook (Czech Republic), using carbon and nitrogen stable isotopes analysis. The results indicated a substantial change of trophic niche and diet of noble crayfish between allopatry and sympatry. In allopatry, both juvenile and adult noble crayfish exhibited a wider trophic niche width compared to juvenile and adult signal crayfish. However, in sympatry, where adult and juvenile noble crayfish coexisted with signal crayfish, their trophic niche width significantly narrowed and their diet shifted towards a more plant-based one. High degree of trophic niche overlap was observed between adults and juveniles in both species, particularly in sympatry rather than in allopatry. The substantial trophic niche overlap and dietary similarity between native noble crayfish and invasive signal crayfish in sympatry, make their long term coexistence on the invaded site unlikely.

Key words: Allopatry, coexistence, diet, invasive species, native species, niche ecology, stable isotopes

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Introduction

Native and invasive species coexistence is a pressing issue in species invasions as new species spreading beyond their natural range usually results in competition with native species. It is often claimed that invasive species are superior competitors over native species with the ability to displace them (Pyšek et al. 2020; Somogyi et al. 2023). However, despite increasing interest in the ecological effects posed by invasive species on native communities, the mechanisms that favour an invasive species to successfully establish in a new ecosystem and coexist with native species are complex and often controversial (Godoy 2019). The success of an introduced species largely depends on its functional traits together with combination of environmental factors and presence of specific biota in given ecosystem (Correia 2002). One of the features that successful invaders typically show, is the presence of a wide trophic niche, a common trait among generalist and omnivorous species, that enables them to exploit a wider range of resources e.g. habitat and food sources, compared to natives (Olsson et al. 2009; Ercoli et al. 2014; Modesto et al. 2021).

There are numerous reports of the reduction and local extinctions of native species after the introduction of new species. Displacement mechanisms by which invaders affect native species can be competition (Hill and Lodge 1994; Westman et al. 2002; Rebrina et al. 2015), reproductive interference (Perry et al. 2001) and transmission of pathogens (Westman et al. 2002; Vilcinskas 2015). However, occasionally native species can survive and coexist with invasive counterparts affecting resources competition (Piscart et al. 2011; Altieri and Irving 2017; Pacioglu et al. 2020; Balzani et al. 2021). Usually when two ecologically similar species occupy the same area, competition can either decrease trophic niche width by decreasing the range of resources used by consumers, or increase it as individuals are forced to consume alternative food sources (Svanbäck and Bolnick 2007; Jackson et al. 2012; Jackson et al. 2016; Copp et al. 2017). However, depending on the level of the total resource exploitation and characteristics of the receiving ecosystem, invasive species can affect the structure of the communities and food webs, leading to unexpected outcomes hindering impact assessment (Jackson et al. 2016; Larson et al. 2017).

Invasive crayfish are becoming more prevalent in Europe (Kouba et al. 2014; Weiperth et al. 2020), which leads to increased encounters and competition with native crayfish, leading to collapses of native populations due to competition for resources or spread of crayfish plague, a severe disease spread by often chronically infected North American crayfish species (Jussila et al. 2021). Previous studies indicate that invasive signal crayfish (*Pacifastacus leniusculus*) may have a wider trophic niche at the species level than native noble crayfish (*Astacus astacus*) living in allopatry, although individual populations have been found to occupy rather similar niches (Olsson et al. 2009; Ercoli et al. 2014), likely due to a wider food sources exploitation, and to different habitat use (Ercoli et al. 2015). Recently Pacioglu et al. (2019) reported coexistence of an invasive spiny-cheek crayfish (*Faxonius limosus*) and native narrow-clawed crayfish (*Pontastacus leptodactylus*) in the lower Danube River, claiming that prolonged competition between species induced resources partitioning between species, potentially making their coexistence possible in the future. Veselý et al. (2021) found that trophic niches of two sympatric invasive crayfish shifted from a full overlapping to a more distinct trophic niche in the presence of a third invasive crayfish species. Furthermore, recent studies have indicated an increase in crayfish resistance, or decreased virulence of crayfish plague, in native European crayfish (Ungureanu et al. 2020; Francesconi et al. 2021; Jussila et al. 2021) which may increase the number of competition-coexistence in the future. However, studies on ecological interactions between sympatric native and invasive crayfish populations are scarce, and more investigations are needed to better understand how and at what degree the ecological behaviour of invasive and native crayfish change when they come to leave in sympatry.

The main aim of our study was to investigate trophic niche and food source use of invasive signal crayfish and native noble crayfish in allopatry, where crayfish species were alone, and in sympatry, where were together, in a local brook. Based on earlier studies (Olsson et al. 2009; Ercoli et al. 2014), we hypothesized that (1) the two crayfish species exploit food sources similarly, and their trophic niches would be wider, with more overlap when in allopatry; (2) in sympatry, the increase in intra- and interspecific competition causes changes in diet, involving the use of different food sources. This results in a lower trophic niche width and partitioning, with low overlap.

Methods

Study area

The study was carried out in September 2018, in Křesánovský brook, Czech Republic. Křesánovský brook is located in the foothills of Šumava mountains near the city of Vimperk (Fig. 1). Large males and females of signal crayfish together with younger cohorts were recorded for the first time in the studied brook in 2015 showing an established population. Size of large crayfish found and local fishermen information suggested that signal crayfish could have been introduced in the brook between 2006 and 2009.

Křesánoský brook is shallow with a maximum depth of 0.6 m (mean depth 0.15 m) and a width ranging from 1.2 m up to 2.5 m in the widest part. The brook is composed of relatively cold water even through the summer season when temperatures usually do not exceed 16 °C (15 ± 1.1 °C). The bottom is covered by pebbles, stones, leaves of deciduous trees, and dead wood, providing many possible shelters for crayfish similar to the burrows in clay-sandy banks at many parts of the brook. Previous fieldwork and local fisherman confirmed the absence of fish in the brook.

The study site can be divided into three sections: allopatric noble crayfish site (only noble crayfish present) located upstream (49°3.90433'N, 13°45.12347'E), sympatric site (both noble and signal crayfish present) (49°3.71333'N, 13°45.30298'E), and allopatric signal crayfish site (only signal crayfish present) (49°3.53700'N, 13°45.65420'E), all of which are separated by small weir and a part of piped stream (approximately 40 m long) (Fig. 1). In general, all sections are fairly similar to each other in terms of habitat type, shelter and food source availability, water current, canopy cover and length (approximately 500 m). However, the allopatric noble crayfish site is partly channelised with concrete structures and probably offers less shelter for crayfish than the other two sites. One of the most important factors for the coexistence of invasive signal and native noble crayfish species is the absence of crayfish plague (*Aphanomyces astaci*), which is deadly for the native species (Westman et al. 2002; Jussila et al. 2021). In the studied brook, recent analyses revealed the absence of crayfish plague (Mojžišová et al. 2020).

Crayfish and food sources sampling

Signal crayfish and noble crayfish, along with their potential food sources (macroinvertebrates and detritus), were collected by kick net and by hand in each site of the studied brook. In laboratory, macroinvertebrates were sorted and identified at the lowest taxonomic level, grouped according to their functional feeding group as collectors, filters, scrapers, shredders and predators, and put into glass tubes, as well as allochthonous detritus to be processed for stable isotope analyses later. Crayfish sex was determined, carapace length (mm) and weight (g) were measured and animals were divided into groups: males and females, juveniles (carapace length < 30 mm) and adults (carapace length ≥ 30 mm). Semi-quantitative macroinvertebrate sampling was conducted using a kick-net in each site to provide additional data on macroinvertebrate food source availability.

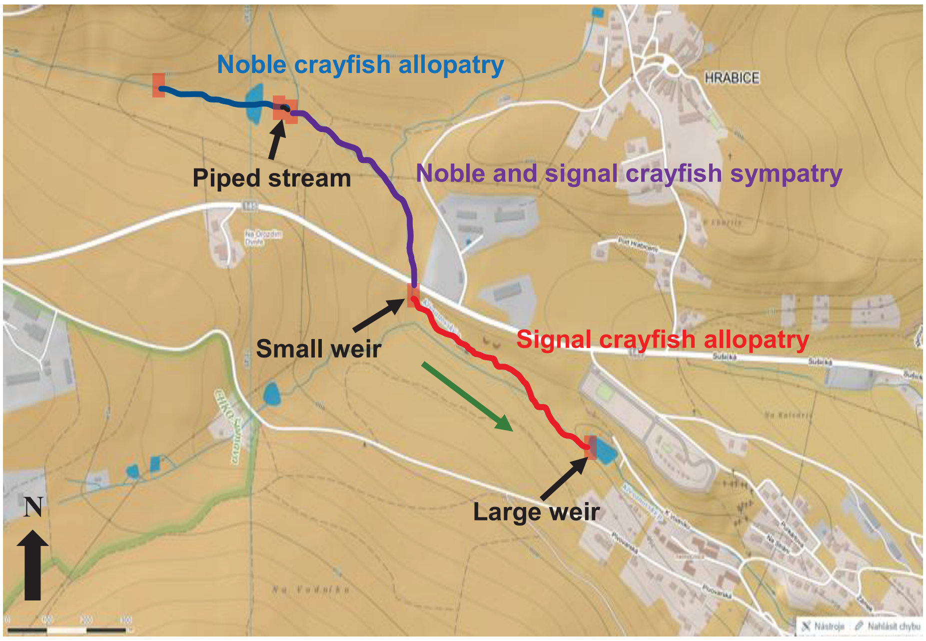

Figure 1. Map of the studied brook indicating different sites in different colours, where crayfish and food sources were sampled. The blue stretch of the brook indicates allopatric noble crayfish site, purple indicates noble and signal crayfish sympatric site, and the red signals crayfish allopatric site. Black arrows indicate weirs, green arrows indicate the flow direction.

Stable Isotopes analyses

All samples were dried in an oven at 60 °C for 48 hours and grinded to a fine homogeneous powder. From animal and plant samples, 0.6 mg and 1.0 mg of materials were weighed respectively into tin caps. All the samples were analysed for carbon and nitrogen stable isotopes with a FlashEA1112 elemental analyser coupled to a Thermo Finnigan DELTAplus Advantage continuous flow isotope ratio mass spectrometer (Thermo Electron Corporation, Waltham, MA, U.S.A.) at Jyväskylä University in Finland. Reference materials used were internal standards of known relationship to the international standards of Vienna Pee Dee, including belemnite for carbon isotopes and atmospheric nitrogen for nitrogen isotopes. Stable isotope ratios are expressed as parts per thousand (‰) delta values relative to the international standards for carbon and nitrogen. White muscle tissue of northern pike *Esox lucius* (Linneaus, 1758) for animal based samples and birch leaves *Betula pendula* for detritus with known isotopic compositions were used as internal working standards to ensure precision of the analyses. One standard sample was run repeatedly after every five samples in each sequence. Standard deviations within reference samples in each sequence were less than 0.1 ‰ for carbon and 0.2 ‰ for nitrogen in pike and in birch leaf samples.

Trophic niche

Crayfish were divided into groups, according to their site of capture and size (juveniles and adults). The trophic niche width of signal crayfish and noble crayfish juveniles and adults, in both allopatry and sympatry were calculated as the Bayesian Standard Ellipse Area (SEA.B; encompassing 95% of the data points) and the corrected standard ellipse area (SEAc; considering 40% of central data points), the latter less sensitive to small sample sizes (Jackson et al. 2011), using the R package SIBER (Stable Isotope Bayesian Ellipses in R) (Jackson et al. 2011). To estimate the degree of trophic niches similarity, the overlap between two ellipses were calculated (Jackson et al. 2019). The overlap is the area, in units of per mil squared (‰^{2}) contained by the shape that lies within the overlapping region, and it is calculated by using the SEAc of each ellipse. Trophic niche overlapping was used to reveal the degree of ecological similarity between adults and juveniles, noble crayfih and signal crayfish populations in allopatry and in sympatry. Trophic niche area of each crayfish group was compared by posterior distribution of paired trophic niches and then calculating their probability of posterior distribution similarity (Jackson et al. 2019). The proportions' range vary from 0, when the ellipses are not overlapping, to 1, when the trophic niches are completely overlapping.

Diet

Collectors, filter feeders and scrapers macroinvertebrate functional groups were grouped to represent one food source (hereafter cfs), due to their similar carbon and nitrogen isotopic values. Proportions of four food sources (shredders, cfs, predator macroinvertebrates and detritus) used by adults and juveniles of both crayfish species living in allopatry and sympatry, were calculated using MixSIAR Bayesian mixing models in R. (Stock and Semmens 2016b; Stock et al. 2018). Crayfish juveniles were also considered as a food source for adults (Veselý et al. 2020). Due to their substantial overlap in carbon and nitrogen stable isotope values showed by MixSIAR model, juvenile crayfish and macroinvertebrate predators sources were pulled together representing one food source (hereafter predator). MixSIAR models were run including the species and age classes, allopatric and sympatric sites as fixed factors selecting residual and process errors (Stock and Semmens 2016a). Fractionation factors for $\delta^{15}N$ and $\delta^{13}C$ were assumed as 3.23 ± 0.41‰ and 0.47 ± 1.23‰ respectively for macroinvertebrate (Zanden and Rasmussen 2001), while for detritus 2.4 ± 0.42‰ for $\delta^{15}N$ and 0.40 ± 0.28‰ for $\delta^{13}C$ (McCutchan et al. 2003). The models were run using Markov Chain Monte Carlo (MCMC) parameters of three chains of 300,000 iterations, burn-in phase of 200,000, and thinning of 100. The percentage contributions of food sources to crayfish diets were generated by the models as posterior distributions with 95% credibility intervals, for each size class and site. Gelman-Rubin and Geweke tests were employed for testing convergence and diagnostic statistics of all model results. For the first test all variables must have values < 1.05 and for the second test means of the first and second part of the chain must be the same.

Statistical analyses

One-way ANOVA was used to test for differences in $\delta^{13}C$ and $\delta^{15}N$ values of food sources between sites. Differences in $\delta^{13}C$ and $\delta^{15}N$ of signal crayfish and noble crayfish between males and females, juveniles and adults, noble crayfish and signal crayfish allopatric sites and sympatric site, were tested using One-way ANOVA. Significant differences (p-value < 0.05) from ANOVAs were further analysed using post-hoc Tukey-HSD pairwise test for comparisons between sites. Fisher's exact test was used to test for differences in shredders, collectors-filters-scrapers (cfs) and predators' macroinvertebrate abundance distributions between noble crayfish allopatric, signal crayfish allopatric and noble and signal crayfish sympatric sites. Assumptions for normality and homogeneity of variances were tested prior to statistical analyses using Shapiro-Wilk and Levene tests, respectively. All statistical analyses and Bayesian models were performed in R (R Core Team 2022).

Results

In total, 87 crayfish, 38 noble crayfish (16 females and 22 males, 15 adults and 23 juveniles) and 49 signal crayfish (19 females and 30 males, 22 adults and 27 juveniles) were caught and analysed for carbon and nitrogen stable isotopes. Density of signal crayfish in allopatry was higher (> 6 individuals per m^2) than noble crayfish in allopatry (< 1 individual per m^2) and noble crayfish and signal crayfish in sympatry sites (> 2 individuals per m^2).

Abundances of shredders, collectors-filters-scrapers (cfs) and predators macroinvertebrate were not different (p-value = 0.56, p-value = 0.35; p-value = 1) between allopatric noble crayfish, allopatric signal crayfish and sympatric noble and signal crayfish sites. In general, each site was dominated by shredders, collectors, filtrators and scrapers, while predators were less abundant (Suppl. material 1).

No significant differences were found in the carbon and nitrogen isotope values of food sources between the three studied sites (p-value > 0.05) (Suppl. material 2). $\delta^{13}C$ values of both crayfish species differed between their allopatric and sympatric sites (p-value = 0.02 for noble crayfish, p-value < 0.001 for signal crayfish) (Fig. 2, Table 1). Noble and signal crayfish muscle tissue $\delta^{15}N$ values differ significantly within and between allopatric and sympatric sites (p-value < 0.001) (Fig. 2, Table 1).

Comparisons between adults and juveniles within sites showed significant differences in $\delta^{13}C$ values in allopatric noble crayfish (p-value = 0.03) and allopatric signal crayfish (p-value < 0.001) sites, while $\delta^{15}N$ values were different only in allopatric signal crayfish (p-value = 0.002) (Fig. 3). However, neither $\delta^{13}C$ nor $\delta^{15}N$ values of signal crayfish and noble crayfish differed significantly between females and males, in any allopatric nor sympatric sites. While noble crayfish and signal crayfish adults showed significant differences in $\delta^{13}C$ values, between allopatric and sympatric sites, juveniles did not exhibit any significant difference among sites (Fig. 4). However, $\delta^{15}N$ values of adults and juveniles of noble crayfish and signal crayfish significantly differ between allopatric and sympatric sites (Fig. 4).

Trophic niches

SIBER model results show that the trophic niche width (SEAc) of adult and juvenile noble crayfish and juvenile signal crayfish shrunk when species were in sympatry (Fig. 5, Table 1). Adult signal crayfish did not show a similar change in trophic niche width, but its position shifted from a lower to slightly higher trophic level and from more depleted to more enriched carbon isotope values (Fig. 5). In allopatry, both species indicated a high interspecific overlap in the trophic niches between adults and juveniles (Fig. 5, Table 2). However, there was little intraspecific overlap in trophic niches when populations were in allopatry (Fig. 5, Table 2). In sympatry, there was a clear increase in both inter- and intra-specific overlap of trophic niches for both species (Fig. 5, Table 2), particularly evident in the signal crayfish.

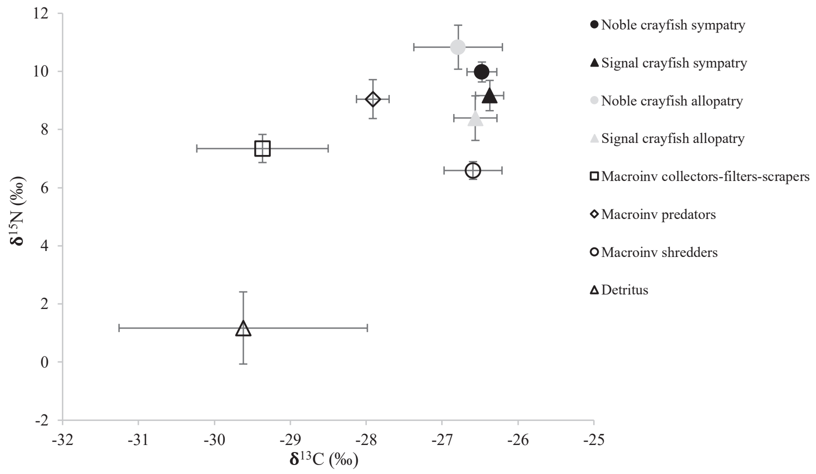

Figure 2. Carbon and nitrogen stable isotopes mean values (± standard deviation) of crayfish groups (noble crayfish and signal crayfish) in allopatry and sympatry and their food sources.

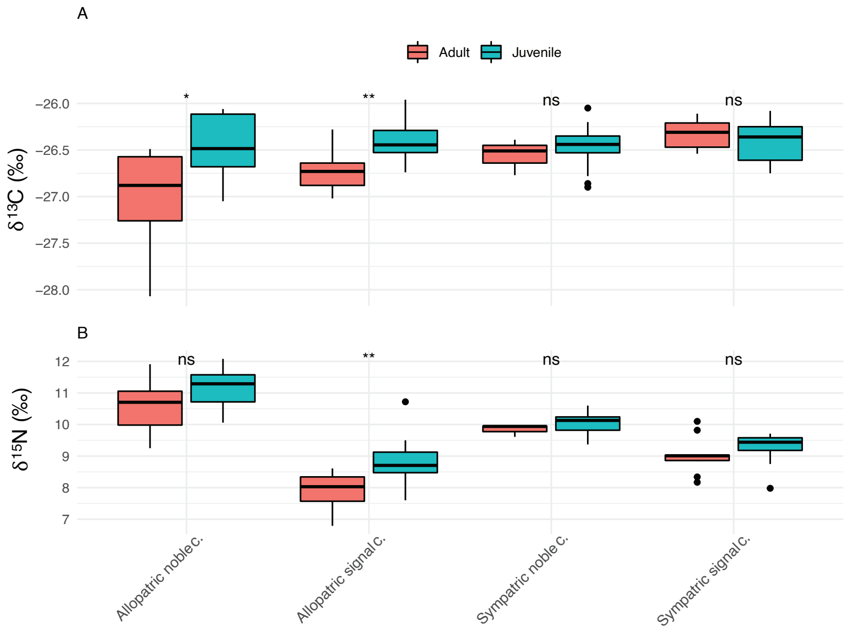

Figure 3. Comparisons of carbon **A** and nitrogen **B** stable isotope values between adults and juveniles within each group of noble and signal crayfish living in allopatry and sympatry. Asterisks (*) (**) and (ns) indicate the significant and not significant difference between the two size classes, respectively.

Table 1. Bayesian standard ellipse area (SEA.B), standard ellipse area corrected (SEAc), carbon and nitrogen stable isotopes mean values (± standard deviation), number of individuals and their length mean values (± standard deviation), divided by group and community.

Community	Group	SEA.B‰^{2}	SEAc‰^{2}	δ^{13}C‰	δ^{15}N‰	N	Carapace length (mm)
Allopatry	Noble crayfish adult	1.09	1.23	-27.04 ± 0.54	10.58 ± 0.74	10	34.99 ± 3.21
	Noble crayfish juvenile	0.78	0.91	-26.48 ± 0.36	11.16 ± 0.63	8	26.20 ± 1.90
	Signal crayfish adult	0.27	0.29	-26.73 ± 0.18	7.94 ± 0.52	13	35.46 ± 2.71
	Signal crayfish juvenile	0.51	0.56	-26.40 ± 0.23	8.82 ± 0.73	14	23.29 ± 1.83
Sympatry	Noble crayfish adult	0.08	0.11	-26.51 ± 0.16	9.83 ± 0.14	5	36.75 ± 4.76
	Noble crayfish juvenile	0.25	0.27	-26.47 ± 0.23	10.02 ± 0.34	15	20.88 ± 4.15
	Signal crayfish adult	0.28	0.32	-26.33 ± 0.15	9.03 ± 0.58	9	42.56 ± 7.86
	Signal crayfish juvenile	0.29	0.32	-26.40 ± 0.21	9.27 ± 0.46	13	18.62 ± 5.18

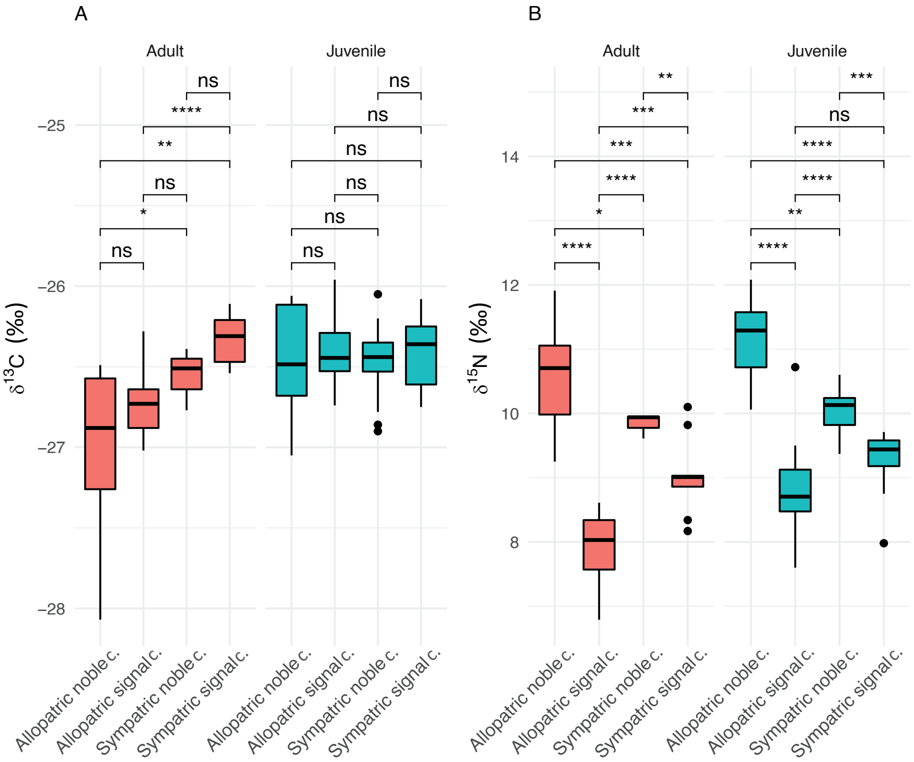

Figure 4. Comparisons of carbon **A** and nitrogen **B** stable isotope values of adults and juveniles between noble and signal crayfish living in allopatry and sympatry. Asterisks (*) (**) (***) and (ns) indicate the significant and not significant difference, respectively.

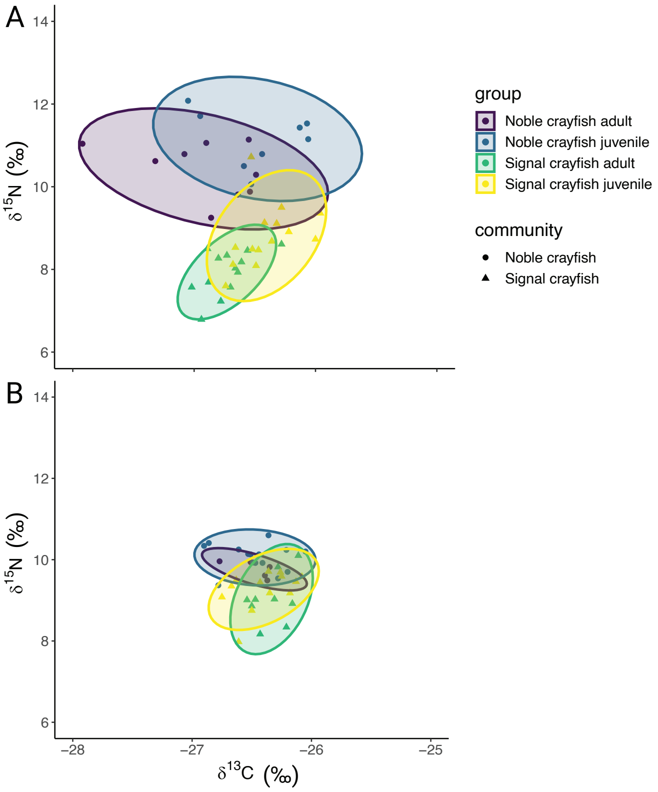

Figure 5. Trophic niche areas, represented by ellipses, of adults and juveniles noble crayfish and signal crayfish in allopatry **A** and sympatry **B**.

Table 2. Proportions of overlapping (%) between each paired group within the two communities.

Community	Group	Overlapping proportions (%)
Allopatry	Noble crayfish adult vs Noble crayfish juvenile	33
	Noble crayfish adult vs Signal crayfish adult	3
	Noble crayfish adult vs Signal crayfish juvenile	20
	Noble crayfish juvenile vs Signal crayfish adult	0
	Noble crayfish juvenile vs Signal crayfish juvenile	12
	Signal crayfish adult vs Signal crayfish juvenile	34
Sympatry	Noble crayfish adult vs Noble crayfish juvenile	37
	Noble crayfish adult vs Signal crayfish adult	19
	Noble crayfish adult vs Signal crayfish juvenile	23
	Noble crayfish juvenile vs Signal crayfish adult	27
	Noble crayfish juvenile vs Signal crayfish juvenile	33
	Signal crayfish adult vs Signal crayfish juvenile	61

Diets

Noble crayfish adults and juveniles used fairly similar proportions of predatory macroinvertebrates in allopatry (47% and 50%) and in sympatry (17% and 19%), while detritus (35% and 38%) and macroinvertebrate shredders (33% and 28%) were used more when in sympatry than in allopatry (Fig. 6A–D, Suppl. material 3). However, signal crayfish adults and juveniles used similar proportions of detritus (42% and 49%) and macroinvertebrates shredder (49% and 39%) in allopatry, while in sympatry, adult and juvenile fed mainly on detritus (60% and 66%) (Fig. 7A–D, Suppl. material 3). However, predatory macroinvertebrates proportions were low in signal crayfish, where allopatric adults and juveniles consumed only 5% and 8% respectively (Fig. 7A, C, Suppl. material 3). Macroinvertebrates cfs source seemed not to be used in the diets of noble or signal crayfish adult and juvenile in allopatry or sympatry (Figs 6A–D, 7A–D, Suppl. material 3).

Discussion

This study indicated a general trophic niche shrinkage of invasive signal crayfish and native noble crayfish when living in sympatry. While only juvenile signal crayfish trophic niche shrink, both adults and juveniles noble crayfish exhibited a remarkable trophic niche shrinkage when in sympatry, decreasing their trophic niche widths, leading to shifts in diets and trophic levels.

Previous studies have indicated that the coexistence of invasive and native species results in trophic niche partitioning, leading to a decrease in niche width and low overlap (Thomson 2004; Jackson et al. 2012; Eloranta et al. 2013; Tran et al. 2015; Balzani et al. 2021) likely due to shifts in diet or habitat. In crayfish, both inter- and intraspecific competition during sympatric coexistence might induce trophic niche partitioning, stemming from differences in use of food sources and/or habitat (Jackson et al. 2014; Jackson and Britton 2014; Pacioglu et al. 2019). Contrary to our first hypotheses, where we expected wider trophic niches and overlap between species in allopatry, our results indicated that trophic niche overlap was higher and niche widths were clearly lower when the species were in sympatry.

In sympatry, crayfish species occupied similar trophic niches which overlapped substantially. A higher crayfish population density in sympatry might have led to

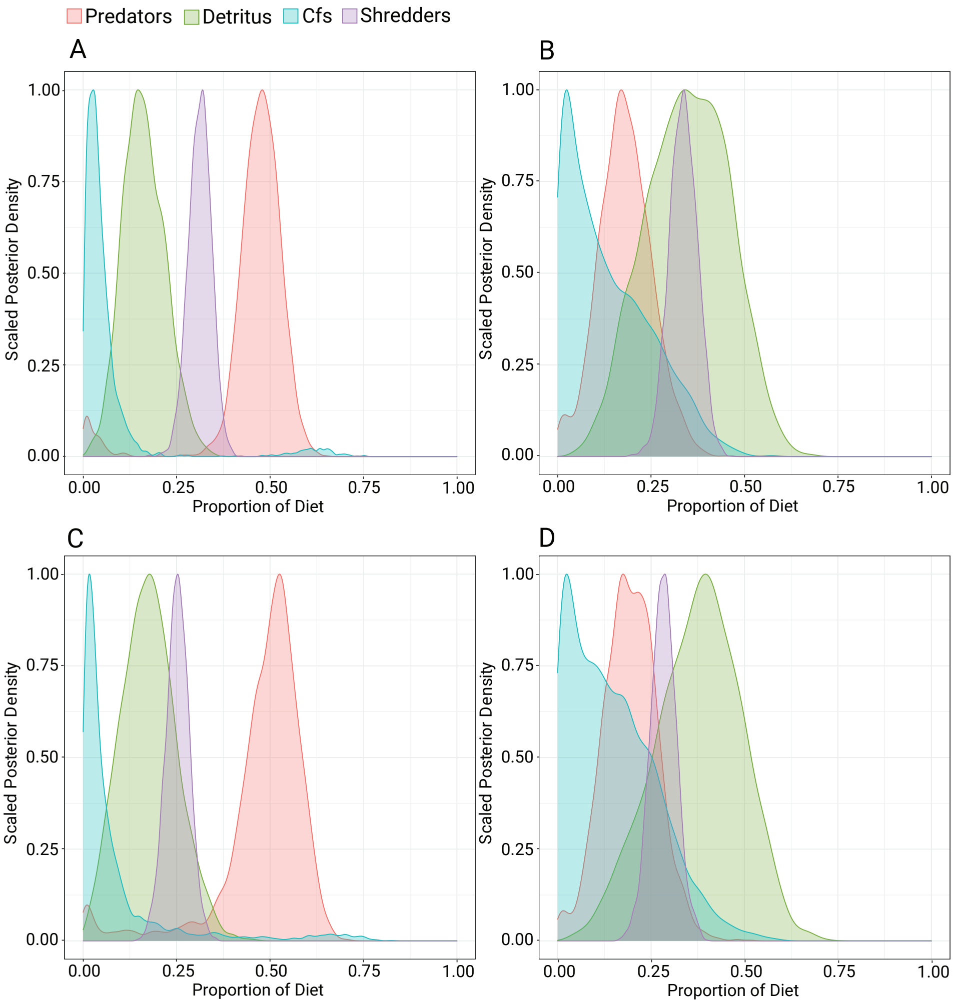

Figure 6. MixSIAR models indicating posterior distribution of food source proportions (predator macroinvertebreates, cfs macroinvertebrates, shredder macroinvertebrates and detritus) used by noble crayfish adults **A** and juveniles **C** living in allopatry, and by noble crayfish adults **B** and juveniles **D** living in sympatry.

increased inter- and intraspecific competition. In our study, sympatric noble crayfish was forced to share habitat and energy sources within a rather restricted habitat, with higher crayfish density, most probably without the possibility of niche partitioning. Larson et al. (2017) investigated the trophic ecology of coexisting invasive crayfish, emphasising the role of the ecosystem on the food web structure and the availability of food sources, subsequently influencing the trophic level of sympatric crayfish species. For instance, Ercoli et al. (2014) found that signal crayfish and noble crayfish trophic niches strongly overlapped and that signal crayfish exhibited a wider trophic niche compared to native noble crayfish, suggesting a wider use of habitat and food resources. Similarly, Olsson et al. (2009) found that signal crayfish occupied a two-fold wider trophic niche than native noble crayfish in Swedish streams. Moreover, they found that high food source availability can also drive a wider trophic niche in crayfish species. Nevertheless, in Ercoli et al. (2014) and Olsson et al. (2009) the comparison of signal crayfish and noble crayfish trophic niches was done between allopatric populations without real coexistence. Our study area is a small brook with a limited amount of habitat, likely the higher density of crayfish in sympatry, along with limited space and food resources sharing, caused decreasing of trophic niche width of noble crayfish and high trophic niche overlap between ecologically rather similar crayfish species.

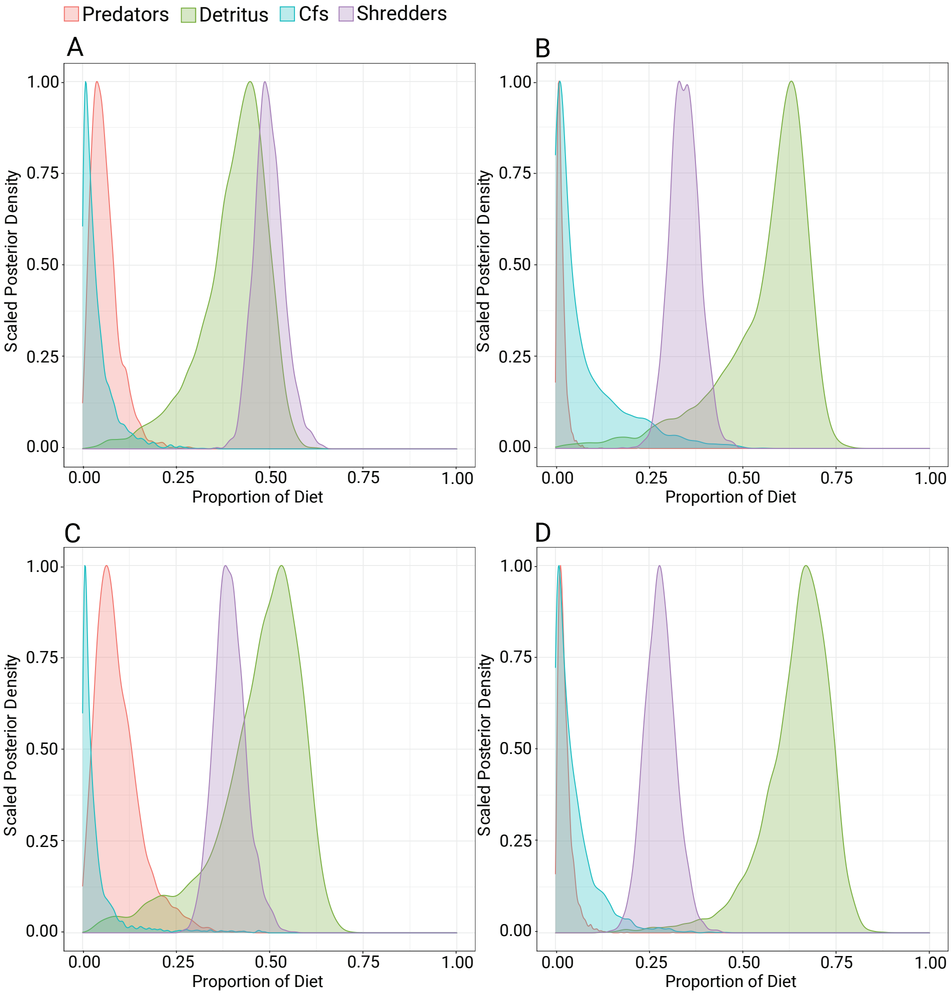

Figure 7. MixSIAR models indicating posterior distribution of food source proportions (predator macroinvertebreates, cfs macroinvertebrates, shredder macroinvertebrates and detritus) used by signal crayfish adults **A** and juveniles **C** living in allopatry, and by signal crayfish adults **B** and juveniels **D** living in sympatry.

Noble crayfish and signal crayfish could have experienced different food source availability between allopatry and sympatry, due to the habitat sharing when in sympatry, which could have influenced their food source use and trophic niches. However, semiquantitative macroinvertebrates data indicated similar macroinvertebrate communities and abundances among the three studies sites (Suppl. material 1), providing similar and abundant food source availability. Yet, riparian habitats and vegetation were similar between sites by visual assessment. These similarities suggest that changes in diets and trophic niches were not driven by differences in food source availability between sites, but rather by the increased competition when crayfish were in sympatry. Ella et al. (2016) highlighted the higher food source consumption generally exhibited by invasive species compared to their native conspecifics. This aspect has also been observed between signal and noble crayfish. In a previous study, Nyström et al. (1999) found that signal crayfish consumed more macroinvertebrate primary consumers and macrophytes than native noble crayfish. Furthermore, results from Hudina et al. (2015) indicated that invasive signal crayfish reduced their aggressive behavior at the invasion front, in sympatry with native conspecific, compared to when the species was in allopatry. In our study, the increased interspecific competition between sympatric invasive and native crayfish species, combined with the high foraging of signal crayfish, likely affected the feeding behavior of native noble crayfish, making the latter less active and changing its predatory behaviour. Ecologically similar invader and native species may highly compete for resources and habitats when in sympatry. Previous studies indicated that intraguild predation (IGP), the interspecific interactions between closely related species that have the ability to prey on each other and on shared preys, can be seen as one of the main drivers in species extinction and replacement (Polis et al. 1989; Holt and Polis 1997). For instance, previous studies on invasive and native Amphipoda species indicate that IGP was the main driver leading to the replacement of native *Gammarus duebeni celticus* by invasive *Gammarus pulex* in nature (Dick et al. 1993, 1999). However, IGP may have direct or indirect effects, such as suppressing competitive conspecifics via direct predation or modifying their ecological behavior, resulting in trophic niche shifts or decreased foraging behaviour (Polis et al. 1989). In our study, results suggest that IGP had indirect effects on native noble crayfish, modifying its trophic niche and diet. The increasing of IGP could also initially increase and then decrease prey density, depending on the strength of the IGP (Chang and Cardinale 2020). In our case, the availability of food sources, such as macroinvertebrate preys, did not differ between allopatric and sympatric sites.

However, the habitat type was slightly different at the noble crayfish site where the brook was more channelised. This may partly explain the observed differences, such as the different foraging behaviour by noble crayfish at this site. Nevertheless, our results suggest that the changes in trophic niche and diet observed in both species were caused by increased competition for resources in a limited habitat rather than the habitat structure itself.

Besides, it is worth noting that interspecific competition and IGP resulting from trophic interactions between invasive and native species can be affected by the presence of disease (Dick et al. 2010). However, in our study, both signal and noble crayfish individuals did not carry diseases like crayfish plague (*Aphanomyces astaci*) or parasites that could have affected our results (Mojžišová et al. 2020).

Our findings showed that trophic niche of adult noble crayfish occupied almost one trophic level higher compared to that of signal crayfish, indicating a different utilisation of energy sources. Larson et al. (2017), in a study conducted in lake ecosystems, interestingly found a higher trophic position of signal crayfish when compared to red swamp crayfish *Procambarus clarkii*, regardless of their occurrence in sympatry or allopatry. However, it is worth noting that in Larson et al. (2017) study, signal crayfish was in its native area while red swamp crayfish was considered the invasive species. The higher trophic level of invasive signal crayfish niche found in Larson et al. (2017), and lower trophic level found in our study, highlight its high trophic behaviour plasticity, which might depend on its native or non native context. Nevertheless, it is important to consider that in Larson et al. (2017) comparisons were made in lake ecosystems with likely more habitats and food sources availability. In a meta-analytical study comparing the trophic niches of invasive fish between native and invaded ecosystems, Comte et al. (2017) highlighted the ability of invasive fishes to shift their trophic niche towards an intermediate trophic position. Moreover, previous studies have suggested that, relying on a diet from a lower trophic level, could make invasive fish more successful in invaded habitats, given the limitless food sources (Gido and Franssen 2007). In our studied brook, signal crayfish might have also exhibited such invasive strategy, owing to its high trophic plasticity.

Trophic niche and diets are known to change according to crayfish sex and size classes (Usio and Townsend 2002; Larson et al. 2010; Veselý et al. 2020). In contrast with results found by Ercoli et al. (2021), in our study, juvenile and adult signal crayfish indicated different carbon and nitrogen isotopic values in allopatry, which shifted juveniles towards more enriched carbon and higher trophic position compared to adults. However, our results indicated that, when in sympatry, both adults and juveniles of noble and signal crayfish underwent changes in trophic positions. Noble crayfish shifted towards lower trophic positions, while signal crayfish towards higher positions changing their diets markedly when in sympatry and suggesting a shift to a more terrestrial-detritus energy source.

Food source use models (MixSIAR) support our trophic niche results, indicating diets of allopatric noble crayfish and signal crayfish generally composed of higher proportions of macroinvertebrate shredders, while in sympatry both species indicated a shift to more detritus-based diets. Predatory macroinvertebrates' use or crayfish cannibalism (predator macroinvertebrate source included also juveniles crayfish) were high in both juvenile and adult noble crayfish living in allopatry. Thus, model results suggest that diets of both size classes of noble crayfish, were protein-based, mostly composed by predator-omnivorous food items. Pacioglu et al. (2019) also found that native crayfish were more carnivorous when they were in allopatry compared to when in sympatry with the invasive crayfish. However, high proportion of detritus and macroinvertebrates shredders fed in sympatry by both size classes and crayfish species, reflected the smaller niche width shifted at lower trophic level, in particular for noble crayfish. The high similarity of diets in sympatric invasive and native crayfish was also found by Pacioglu et al. (2019).

Conclusions

The trophic niche overlap and diet similarity of native noble crayfish and invasive signal crayfish in sympatry, coupled with limited availability of alternative resources and habitats, make their long term coexistence in Křesánovský brook unlikely, thereby posing a threat to noble crayfish (Westman et al. 2002). Although the eradication of invasive species is challenging, efforts to control the further spread of signal crayfish, and the restoration of the native noble crayfish population, could help preserve the native species (Perales et al. 2021). Additional research is needed to address the knowledge gap concerning the ecological interactions and responses of native and invasive species when living together. This might include assessing the specific response of interacting species and making comparisons across different ecosystems over an extended period.

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 FE, TR, LV. Investigation FE, TR, MB, AK, MB and LV. Formal Analysis FE and TR. Writing initial draft FE, TR and LV. Revision and approval of the final submission FE, TR, MB, AK, MB and LV.

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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

Semiquantitative macroinvertebrates representing eudominant and dominant species (++), sub- dominant and recedent species (+) and sub-recedent species (-)

Authors: Fabio Ercoli, Timo J. Ruokonen, Martin Bláha, Antonín Kouba, Miloš Buřič, Lukaš Veselý

Data type: pdf

Link: https://doi.org/10.3897/neobiota.98.127329.suppl1

Supplementary material 2

Carbon and nitrogen stable isotopes mean values (standard deviation) and number of samples (n) of food sources in each site

Authors: Fabio Ercoli, Timo J. Ruokonen, Martin Bláha, Antonín Kouba, Miloš Buřič, Lukaš Veselý

Data type: pdf

Link: https://doi.org/10.3897/neobiota.98.127329.suppl2

Supplementary material 3

Posterior distribution represented by mode (50%) and confidence interval (2.50% and 97.50%), of food sources used by noble crayfish and signal crayfish adults and juveniles in allopatry and sympatry

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Research Article

Developing best management practices for the invasive monk parakeet (*Myiopsitta monachus*) in urban environments

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Abstract

Managing invasive species has become a major environmental challenge due to their global ecological and socioeconomic impacts. Prioritising effective strategies is essential, especially given the often limited funding. Data from real control programmes are crucial for developing long-term management plans. Between May 2021 and April 2023, the City of Madrid implemented a control plan to reduce its monk parakeet (*Myiopsitta monachus*) population. This study assessed: (1) the plan's efficacy, (2) the impact of removing different age classes on population viability, (3) the capture efficacy and cost-efficiency of various capture methods, (4) the optimal combination of capture methods and (5) the effectiveness of different baits in attracting parakeets. The plan eliminated approximately 87% of the juvenile and adult population present at the plan's onset, reducing total population projections for 2023 and 2031 by 50%. Projections indicated that maintaining the second-year removal intensity for three additional years could have nearly eradicated the species. Removing juveniles and adults proved more than twice as effective at curbing population growth as targeting eggs and nestlings, leading to prioritising juvenile and adult removal year-round. Of the five methods used – shooting, folding net, hand-held net launcher, egg culling and nestling culling – shooting was the most effective in reducing population growth both in and outside spring. Optimisation models suggested prioritising shooting and the combination of folding net and net launcher in spring and exclusively the latter outside spring. We recommend using the shooting method year-round, complemented by the combined use of the folding net and net launcher outside of the spring season. Bread and a mixture of bread, apple and parrot feed were the most effective and cost-efficient baits attracting parakeets, with bread recommended for simplicity. This study enhances understanding of parrot management strategies, offering insights for more effective and cost-efficient control of invasive monk parakeet populations.

Key words: Alien species, bird capture methods, bird control, egg culling, exotic species, feeding preferences, nestling culling, parrots

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Introduction

Biological invasions by Invasive Alien Species (IAS) are considered a major environmental issue due to their significant ecological (Wilcove et al. 1998; Levine et al. 2003) and socioeconomic impacts worldwide (Eiswerth and Johnson 2002; Pimentel et al. 2005; Diagne et al. 2021; Haubrock et al. 2021; IPBES 2023). In the 20th and 21st centuries, the spread of IAS has reached unprecedented levels, facilitated by increasing long-distance trade (Meyerson and Mooney 2007). Although not all introduced alien species become invasive, the rates of invasions are growing and expected to continue accelerating in the coming decades (Seebens et al. 2017, 2021). Consequently, the impacts of IAS are magnified, making their management a major global challenge.

The management of IAS involves a wide range of actions in environmental policy and practice, including preventing introductions, containing or eradicating new spreads and mitigating the impacts of established populations (Simberloff et al. 2013). These actions are essential for biodiversity conservation and protecting economic interests, ecosystem services and human health (Crowley et al. 2017). However, given the high costs of IAS management and often limited funds (Tempel et al. 2004), it is critical to prioritise effective management strategies in IAS control plans. Unfortunately, the results and lessons learned from IAS control programmes are often poorly reported, residing mainly within grey literature and lacking solid statistical analysis to support the conclusions (Martins et al. 2006; Brooke et al. 2007; Holmes et al. 2015; Iacona et al. 2018; Avery and Feare 2020). The lack of adequate evaluation of what works and what does not can prevent managers from effectively implementing control and eradication measures. Moreover, failing to check whether management works weakens the arguments for further investment (Sutherland et al. 2004). Therefore, obtaining data from real control programmes is essential for developing more adaptive and effective long-term management strategies.

Amongst vertebrates, the monk parakeet *Myiopsitta monachus* is one of the most widely distributed and successful IAS worldwide (Calzada Preston and Pruett-Jones 2021). From the 1970s onward, the popularity of this parrot as a pet increased greatly, with millions of individuals captured and exported from South America (its native habitat) to North America and Western Europe (Domènech et al. 2003; Strubbe and Matthysen 2009; Postigo et al. 2017; Souviron-Priego et al. 2018). Deliberate releases and accidental escapes have led to the rapid spread and establishment of increasingly large breeding populations of this species, particularly in urban environments (Da Silva et al. 2010). The monk parakeet has established populations in 26 countries (Calzada Preston et al. 2021), with the highest rates of population growth observed in the USA (Van Bael and Pruett-Jones 1996; Burgio et al. 2016), Mexico (Hobson et al. 2017), Israel (Postigo et al. 2017) and across the United Kingdom, Greece, Italy and Spain (Postigo et al. 2019). Spain currently hosts the largest invasive population of monk parakeets in Europe, estimated at up to 21,000 individuals as of 2015, with 40% residing in Madrid (Molina et al. 2016). In 2013, Spanish legislation classified the monk parakeet as an IAS, which prompted the initiation of prevention measures and the development of strategic management plans, with local and regional governments tasked with implementation.

Between May 2021 to April 2023, the City of Madrid implemented a management plan aimed at reducing its monk parakeet population, acknowledging that complete eradication was unattainable and that a small residual population would likely persist. To carry out this initiative, the Madrid City Council contracted "Mantenimiento de Infraestructuras S.A.U." (hereafter, MATINSA), which employed five common bird control methods to capture parakeets: shooting, folding nets, hand-held net launchers, egg culling and nestling culling. The capture efficacy and cost-efficiency of these capture methods were evaluated through a collaborative study between Universidad Rey Juan Carlos and MATINSA. The specific objectives of the study were to: (1) assess the impact of the management plan on the population viability of the monk parakeet; (2) examine whether the removal of individuals from different age classes has differential effects on population viability; (3) identify the most effective capture method (i.e. highest capture rate per hour) and the most cost-efficient one (i.e. lowest cost per parakeet captured), while considering seasonal variation; (4) determine the combination of control methods that maximise effectiveness and cost-efficiency; and (5) determine which bait type attracts the highest number of parakeets per hour and offers the best cost-efficiency (i.e. lowest cost per parakeet attracted). This study aims to enhance understanding of the effectiveness of current management tools to facilitate more effective and cost-efficient management of this species in the future.

Methods

Study species

The monk parakeet (*Myiopsitta monachus*) belongs to the order Psittaciformes (commonly known as parrots) and is native to temperate and subtropical South America. Monk parakeets are unique amongst Psittaciformes for building nests rather than nesting in cavities. They often build communal nests, where multiple pairs collaborate in constructing and using the nest year-round for roosting and breeding (Bucher et al. 1991). They can live up to 15 years, though this lifespan applies to captive birds (Burgio et al. 2020). In the wild, their lifespan is estimated to be around 13 years (Senar et al. 2021). Reproductive efficiency of monk parakeets in their invasive range is higher than in their native range: in the invasive range, 55% of first-year parakeets breed compared with almost zero in South America (Bucher et al. 1991; Martín and Bucher 1993; Senar et al. 2019). Fledging success during the first brood is double in the invasive range (3.3 ± 1.08 vs. 1.6 ± 0.53) (Navarro et al. 1992; Peris and Aramburú 1995; Senar et al. 2019) and the percentage of pairs attempting second broods is three times higher compared to the native range (56 ± 9.93% vs. 15%) (Navarro et al. 1992; Senar et al. 2019). Invasive monk parakeet populations exhibit exponential growth rates, with populations in Mediterranean countries experiencing higher exponential growth, greater spread rates and more rapid colonisation than those in the Atlantic Region (Postigo et al. 2019). Population growth rate estimations in Spain ranges from 0.15 to 0.31 depending on the area studied and the year, with a population doubling time of 4.8 to 2.3 years (Muñoz 2003; Molina et al. 2016; Postigo and Senar 2017; Postigo et al. 2019; Senar et al. 2021).

Various negative impacts of the monk parakeet have been reported throughout both its invasive and native range, including damage to crops (Batllori and Nos 1985; Tillman et al. 2000; Conroy and Senar 2009; Senar et al. 2016; Muñoz-Jiménez and Alcántara-Carbajal 2017; Battisti 2019; Battisti and Fanelli 2022; Castro et al. 2022) and vegetation (Shields et al. 1974; Batllori and Nos 1985; Menchetti and Mori 2014), risk of nest fall (Esteban 2016), interspecific aggression (Batllori and Nos 1985; Weiserbs and Jacob 1999; García and Bonfil 2007; Dangoisse 2009; Briceño et al. 2019), spreading of exotic plants (Blanco et al. 2015, 2016, 2018; Tella et al. 2015; Hernández-Brito et al. 2021a), facilitation of the establishment of introduced and invasive birds (Briceño et al. 2019; Hernández-Brito et al. 2021b, 2022) and the introduction and spread of parasites and pathogens (Aramburú et al. 2003; Mori et al. 2015, 2018, 2019; Briceño et al. 2017, 2023; Ancillotto et al. 2018; Martínez-de la Puente et al. 2020; Morinha et al. 2020; Sandoval-Rodríguez et al. 2021; Blanco-González et al. 2024).

Study area

The City of Madrid covers an area of 604 km^2 and is home to 3,332,000 inhabitants, with a population density of 5,300 inhabitants/km^2. The city has an average altitude of 657 metres and a continental Mediterranean climate, characterised by mild, wet winters and warm, dry summers. Madrid boasts approximately 6,400 hectares of publicly-owned municipal green areas and an estimated total of 16,700 cedars (Morcillo San Juan 2015), including both Himalayan cedars (*Cedrus deodora* Roxb. ex D.Don) and Atlas cedars (*Cedrus atlantica* Endl.). These trees are the most favoured by monk parakeets for nest construction in Madrid (Martín-Pajares 2006; Molina et al. 2016). Most parakeet capture efforts were conducted on public land, except for shooting, which was primarily carried out on private land due to regulatory restrictions and where conditions allowed. Nest removals were also performed, mainly on public land, but occasionally on private property when there was an imminent risk of nest collapse.

Censused population of the monk parakeet in Madrid

At the outset of the management plan, the most recent census of monk parakeets in Madrid, conducted in 2019, estimated a population of 11,156–12,967 reproductive individuals distributed across 4,418 nests and 9,402 chambers (Nebreda et al. 2019). This indicated a population doubling time of approximately 4.4 years compared to the 2015 census, which recorded 6,291–7,113 individuals, 2,198 nests and 4,945 chambers (Molina et al. 2016). Both censuses used consistent methodology, counting nests and chambers and estimating occupancy rates based on the average number of resident individuals per chamber, with data collected from the ground using binoculars. These surveys were conducted at the same time of year, largely by volunteers.

Based on the 4.4-year doubling time, the monk parakeet population in Madrid at the start of the control plan in spring 2021 was estimated at 15,360 –17,855. This closely aligns with MATINSA's estimate of 15,177 – 17,580 individuals across 4,667 nests and 12,645 chambers at the plan's outset (MATINSA unpublished data) (Fig. 1A). MATINSA retrospectively calculated these figures in August 2022, following the removal of 2,040 nests and a total count of 5,538 chambers. MATINSA's calculated chamber-to-nest ratio was 2.71, compared to 2.13 from the 2019 census (Nebreda et al. 2019). An advantage of MATINSA's census was its direct chamber counts at the nests using aerial platforms, likely providing a more accurate estimate than previous ground-level surveys (Molina et al. 2016; Nebreda et al. 2019). However, MATINSA did not independently estimate the average occupancy per chamber, instead applying the occupancy rate from Nebreda et al. (2019), which ranged from 1.2 to 1.39 individuals per chamber.

It is important to highlight that a post-control census was not conducted at the conclusion of the management plan. As a result, the population size at the end of the management plan was estimated using demographic projections.

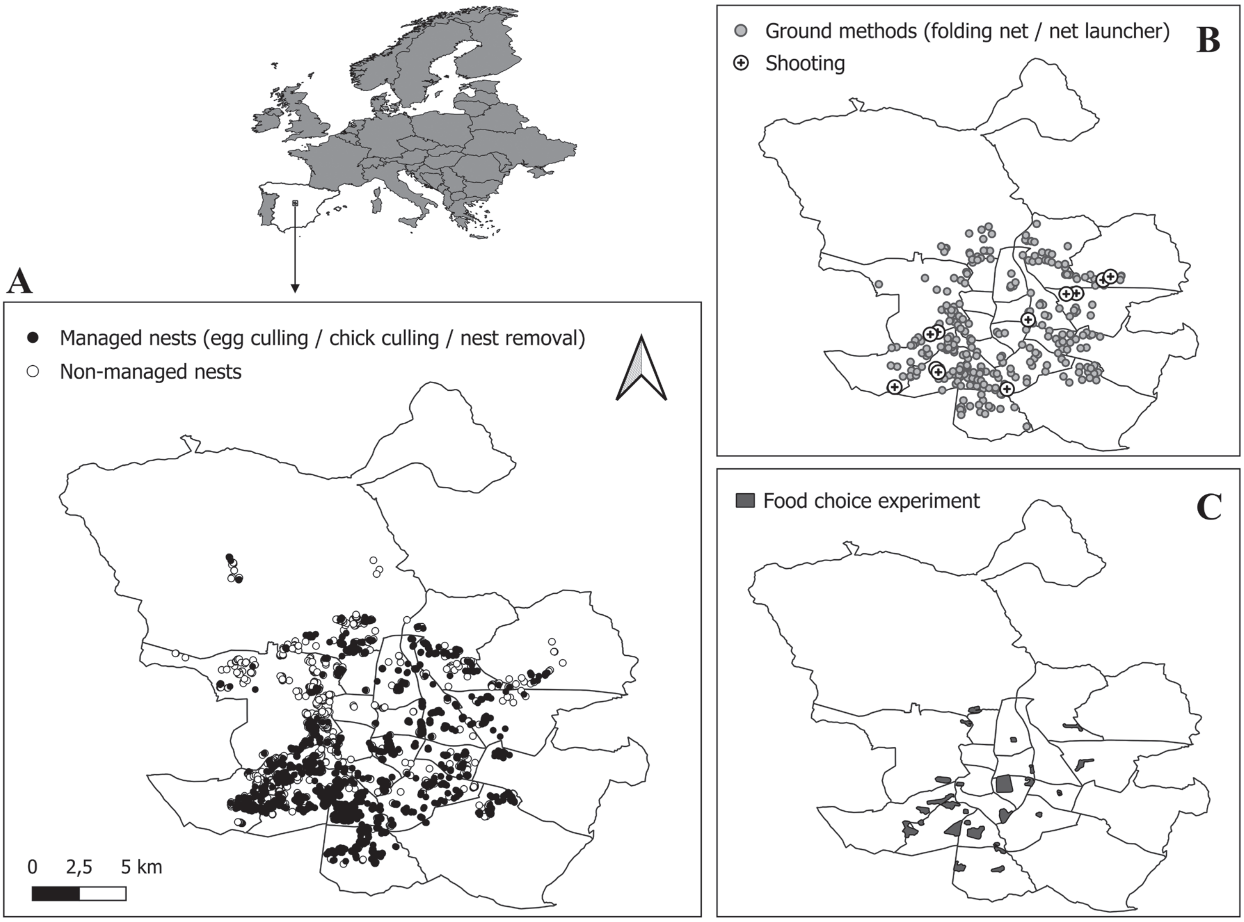

Figure 1. Map showing the location of the City of Madrid in Spain. **A** Ahows the location of the monk parakeet nests surveyed by MATINSA, distinguishing between nests managed during the control plan and those that were not managed **B** shows the sites where captures were made using ground methods and shooting **C** shows the parks where the food choice experiment was conducted.

Capture methods and lethal control

Captures were performed by 10 capture teams, each typically composed of two individuals, working Monday to Friday from 7:00 to 15:00 h. Different capture methods were employed, categorised into three main groups: shooting (aimed at birds perched on high branches, never on the ground), ground methods (conducted at ground level using net capture techniques) and aerial methods (accessing the nests using an aerial work platform). Below, we provided detailed information on each capture method:

Shooting: Skilled shooters employed pre-charged pneumatics (PCP) air rifles (5.5 mm diameter) equipped with telescopic scopes. The rifles fired a single round-tipped projectile per shot aimed to eliminate the targeted individual. The rifle's usage was confined to specific parks with restricted access and to private areas (Fig. 1B).

Ground methods: 1) Folding nets: Nets were discreetly installed at ground level to avoid detection by parakeets. Parakeets were attracted by bait placed on the net. Upon landing, the net was remotely activated, effectively immobilising the parakeets on the ground. Successful execution of this method required both the rotation of capture sites and pre-baiting the area for several days (on average 8.0 days ± 2.5), allowing the parakeets to become familiar with the location and thereby enhancing the potential for capturing multiple birds in each operation; 2) Hand-held net launcher (hereafter, “net launcher”): This portable CO_2-powered net launcher features a net size of 1 m × 1 m and was capable of capturing a few individuals at most (average: 1.5; range: 1–6), but its discretion and quick setup allowed for multiple attempts per day (Fig. 1B).

Aerial methods: 1) Egg culling: Since nest removals were also performed as part of the control plan, eggs were removed when the nests were being taken down. In cases where the nests were not being taken down, the eggs were pricked to interrupt the asepsis inside and to fatally damage the embryos (Smith et al. 1999). This strategy seeks to deceive the parents into maintaining incubation, thereby preventing them from laying replacement eggs; 2) Nestling culling: Parakeet nestlings were removed regardless of whether the nest was meant for removal or not. Since there were few days when only nestlings were culled, this method has been analysed in conjunction with egg culling, as it was common to combine both egg and nestling culling on the same day (Fig. 1A).

All parakeets captured alive, either by ground or aerial methods, were transported to an authorised veterinary clinic for lethal control. Birds were individually euthanised by a veterinarian on the same day of capture. At the time of euthanisation, birds were under anaesthesia (Isoflurane 100%; 2 l/min-5%). An injection of 2 ml of pentobarbital sodium (200 mg/ml) was administered at the base of the neck between the insertion of the pectoralis and supracoracoideus muscles. Verification of the cessation of respiratory signs and the disappearance of reflexes confirmed successful euthanasia. In accordance with the law, the carcasses of all parakeets (both captured-euthanised and shot) were cremated.

Notably, the capture of monk parakeets faced public opposition at various stages, particularly in the initial phases. However, these events did not significantly disrupt the management plan (Appendix 1).

Food choice experiment

The success of capturing monk parakeets with nets improves when baits are used to attract them to the ground. During the management plan for the monk parakeet in Madrid, four different baits were used interchangeably: stale bread (hereafter, “bread”), apple, commercial parrot feed (Pilesan® brand; hereafter, “parrot feed”) and a mixture of all these foods (hereafter, “mixture”). To determine which type of bait attracts the most parakeets to the ground, we conducted a food choice experiment in 27 parks in Madrid City where parakeets were present (Fig. 1C). In three parks, we performed the experiment twice, separated in time by a minimum of 12 days to prevent learning by parakeets, adding a total of 120 observations (n = 30 replicates × 4 baits per replicate).

The experiment was conducted from late January to mid-March 2023, between 7:00 and 10:00 h. In each park, a 1 m x 1 m area was selected to place the baits on the ground (hereafter, “feeder”), ensuring that all feeders were similar in terms of spatial structure (e.g. presence of trees and paths). Each feeder contained a single type of bait (bread/apple/parrot feed/mixture). The food in each feeder was spread out evenly to facilitate parakeet detection, with larger portions of bread and apple used to deter parakeets from carrying them away. Each feeder was supplied with one kilogram of food (the mixture bait contained approximately 0.3 kg of bread, 0.3 kg of apple and 0.3 kg of parrot feed). Baits were not replenished during the experiment. Each feeder was placed approximately 20 m from the observers, with an angle of 45° between adjacent feeders, forming a cross (Appendix 2). The relative positioning of the feeders was randomised daily. Observations were made by two observers stationed at the centre of the cross, starting from the arrival of the first parakeet at any of the four feeders. From that moment onwards, each observer recorded the number of parakeets entering two adjacent feeders at 5-minute intervals over a two-hour period. Every 5 minutes, observers would alternate between their feeders to avoid biases. Each parakeet entering the feeder was counted, regardless of whether it had previously visited that feeder or not. After the experiment concluded, any remaining food was saved for reuse or properly disposed of.

Data analyses

To assess the impact of the management plan and the removal of individuals from different age classes on the population viability of the monk parakeet, we conducted demographic projections incorporating both demographic and environmental stochasticity using the "Rramas" package (De la Cruz Rot 2019). We defined ten age classes: nestling, first-year juvenile and eight adult classes ranging from second-year birds to individuals over eight years old. Mean reproduction and survival parameters, along with standard deviations for each age class, were obtained from Senar et al. (2019, 2021) to construct two transition matrices: one with average values and another incorporating standard deviations to account for environmental stochasticity (Appendix 3). The egg stage was excluded from the model, as age transitions were equated to calendar years, omitting intra-annual transitions such as the shift from egg to nestling. Consequently, the nestling age class included both eggs and recently-hatched chicks.

Using these parameters, we evaluated the impact of MATINSA's management plan on population viability (Objective 1) by estimating the effect on population size following the removal of individuals during the first and second years of the control plan (12 months each). These results were compared against two scenarios: (1) no management actions and (2) a continuation of management beyond the two years of the actual plan, assuming the removal of individuals at the same rate as in the second year until virtual eradication was achieved.

All projections were modelled over a 10-year timeframe. The baseline scenario (no management) was compared with the results obtained from the demographic analysis of Senar et al. (2021) to validate the accuracy of the population modelling.

To assess whether the removal of individuals from different age classes had differential effects on population viability (Objective 2), we estimated the impact of annually removing a fixed number of either eggs and nestlings or juveniles and adults on the annual population growth rate over a 10-year period. Population growth rate was calculated as:

$$\text{Population growth rate}(\%) = \left(\left(\frac{N_{t10}}{N_{t0}}\right)^{\frac{1}{t_{10}}} - 1\right) \times 100$$

where N_{t10} is the population size at the end of the 10-year period, N_{t0} is the initial population size at the beginning of the study and t_{10} represents the duration of the period in years (10 years in this case).

The "Rramas" package used for Objectives 1–2 allowed for the direct inclusion of the number of eggs, juveniles and adults removed annually.

We then analysed whether capture methods differed in terms of: (1) capture efficacy, measured as the number of parakeets captured per hour (referred to as "birds/h"); and (2) cost-efficiency, measured as the cost per parakeet captured (referred to as "€/bird") (Objective 3). We assessed these measures during two different seasons: the "spring" (21 March – 21 June) and the "non-spring" period (rest of the year; 22 June – 20 March). This division reflects the primary nesting season occurring during spring, allowing us to evaluate method performance both within and outside of this period. This division also provides an intuitive calendar for the implementation of control methods in future management plans.

For hourly parameterisation, we defined a standard working day as 8 hours. Typically, each team consisted of two individuals, though sometimes they could be formed by only one person. Therefore, we set the working days for two-person teams at 16 hours and for one-person teams at 8 hours. On certain days, capture techniques were combined with non-capture activities, such as nest removal and baiting of feeders. Consequently, we also included the time invested on these extra activities when calculating the total time needed for each capture method. Additionally, we computed costs associated with personnel, vehicle rental and petrol, incineration of corpses and material used for capture activities and at the veterinary clinic. Detailed explanations of these calculations can be found in Appendix 5.

For Objective 3, we compared the capture methods (shooting, folding net, net launcher, egg culling, nestling culling) including two combinations of methods frequently used: the combination of folding net and net launcher and the combination of egg and nestling culling.

To test whether the capture method and the season influenced capture efficacy, we fitted a generalised linear mixed model (GLMM) with a negative binomial error distribution (nbinom1) and a logarithmic link function. The response variable was the number of captured parakeets. We included the interaction between method and season as fixed factors and team as a random factor. The capture effort, measured as the number of hours dedicated to a specific capture method, was adjusted using the "offset" argument, as the time allocated by different teams to each capture method varied daily.

To test whether the capture method and the season influenced cost-efficiency, we fitted a GLMM with a Gaussian error distribution and a logarithmic link function. The response variable was the cost of capturing a single parakeet. We included the interaction between method and season as fixed factors and team as a random factor. In both models, we incorporated a temporal autocorrelation using the Ornstein-Uhlenbeck covariance structure and corrected the heteroscedasticity within factor levels using the "dispformula" argument. However, we were unable to fully correct the heteroscedasticity issue, likely due to lack of orthogonality in the data since the design was not fully crossed (i.e. not all teams conducted all control methods in each season).

To determine the optimal combination of capture methods that maximise effectiveness and cost-efficiency (Objective 4), we utilised the population dynamics and decision-making model developed by Senar et al. (2021). First, we evaluated the individual impact of each control method on population growth rate based on their capture efficacy. For this analysis, all control methods were considered, with an annual effort of 2,000 hours per method, a feasible number of hours as determined by the control plan. Second, we identified the combination of methods and effort levels that maximised population reduction, while minimising associated costs. For computational efficiency, only the three most effective methods were included: shooting, the combination of egg and nestling culling and the combination of folding net and net launcher. The maximum annual effort for each method was capped at 2,000 hours. Analyses were conducted separately for spring and non-spring periods.

To investigate the influence of bait type on the number of attracted parakeets (objective 5), we fitted a GLMM with a negative binomial error distribution (nbinom1) and a logarithmic link function. The response variable was the number of parakeets attracted per hour, with the type of bait as a fixed factor and park as a random factor. To determine the most cost-effective bait (lowest investment per attracted parakeet), we fitted a GLMM with a Gaussian error distribution and a logarithmic link function. The response variable was the cost of attracting a single parakeet, with the type of bait as a fixed factor and park as a random factor. In both models, we incorporated a temporal autocorrelation using the Ornstein-Uhlenbeck covariance structure.

Models for objectives 3 and 5 were fitted using the "glmmTMB" package (Brooks et al. 2017). Model selection followed the recommendations of Zuur et al. (2009), with the suitability of different models evaluated, based on the Akaike Information Criterion (AIC). Models with a difference in AIC > 2 indicated that the worst model had virtually no support and could be omitted. If there were more than one best model (difference in AIC ≤ 2), we selected the simplest model (Wagenmakers and Farrell 2004). Collinearity between predictors was assessed using the "performance" package (Lüdecke et al. 2021) and model assumptions were checked using the "DHARMa" package (Hartig 2022). To test for significant differences amongst the levels of predictor variables (i.e. either capture methods or types of baits), we conducted Tukey's and Sidaks's *post-hoc* pairwise comparisons tests from the "emmeans" (Lenth 2024) package. We used Tukey's test when comparing all pairs of predictors with equal sample sizes and Sidak's test when comparing specific pairs of predictors with unequal sample sizes. Goodness-of-fit of the models was assessed using Efron's pseudo-R^2 from the "performance" package. All statistical analyses were performed using R Statistical Software v. 4.2.2 (R Core Team 2022).

Results

Effects of management on population viability

A total of 14,321 adult individuals (including both adults and juveniles, as distinguishing between them was not possible), 2,822 nestlings and 2,062 eggs were eliminated over the two years of the management plan. During the first year, MATINSA culled 1,560 eggs and nestlings and 4,535 juveniles and adults. In the second year, 3,324 eggs and nestlings and 9,786 juveniles and adults were removed (see Table A3 in Appendix 4 for monthly capture numbers).

The population viability analysis projected that, by the end of 2023 and following the two-year management plant, the monk parakeet population would consist of 18,153 individuals, including nestlings, juveniles and adults. In the absence of management, however, the population would have reached 36,138 individuals by 2023, 99% greater than under the management scenario. By 2031, ten years after the plan's initiation, projections estimated the managed population at 71,636 individuals, compared to 155,534 individuals without intervention, 117% greater

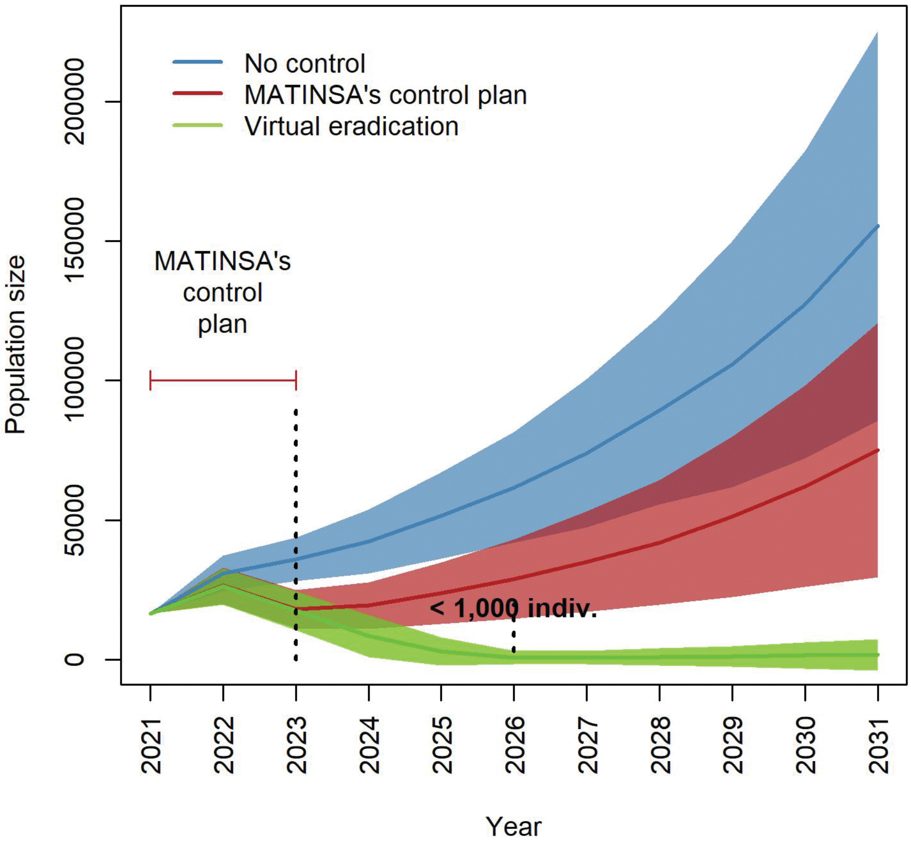

Figure 2. Projected monk parakeet population dynamics in Madrid over a 10-year period. The red line represents the population trajectory under the management plan implemented from 2021 to 2023, while the blue line shows the projected population trajectory without management intervention. The green line depicts a management scenario targeting a reduction of the adult population to fewer than 1,000 individuals. Shaded areas represent the (demographic and environmental) uncertainty range around each projection. The dashed vertical line marks the population size at the end of the management plan. Population size is measured as the combined total of nestlings, juveniles and adults. Models do not assume a carrying capacity, which would otherwise result in predictions reaching an asymptote as the population growths.

than under the management scenario (Fig. 2). The growth rate (r) between 2023 and 2031 was estimated as 0.16 for the managed scenario and 0.25 for the unmanaged scenario, corresponding to population doubling times of 4.33 and 2.77 years, respectively. To reduce the adult population to fewer than 1,000 individuals, an additional three years of management would have been required, assuming the removal rates achieved in the second year were sustained annually. This reduction would likely have been accomplished by 2026.

Examining the hypothetical impact of management strategies, based on age class, our models indicated that removing 1,000 eggs and nestlings annually over a 10-year period would reduce the annual population growth rate by approximately 2.1%, compared to the scenario without management. In contrast, removing 1,000 juveniles and adults annually over the same period resulted in a more substantial reduction of 7.4%. To achieve a neutral annual growth rate (0%) over 10 years, the model suggests removing either 4,800 juveniles and adults or 11,000 eggs and nestlings (Fig. 3). Sustained removal of more individuals than the indicated thresholds annually over 10 years would result in a negative growth rate, leading to a gradual population decline.

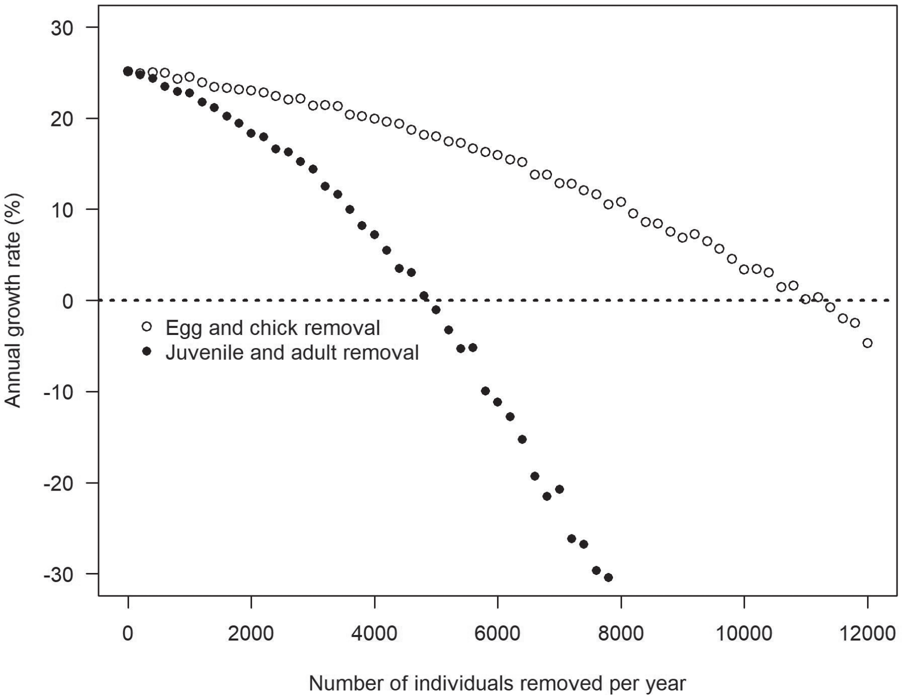

Figure 3. Relationship between the number of individuals removed per year and the annual population growth rate (%) of the monk parakeet after 10 years of simulation. Two management scenarios are compared: egg and nestling removal (open circles) and juvenile and adult removal (closed circles). A positive growth rate indicates population increase, a negative rate indicates decline and a neutral rate (0%) reflects no net change in population size.

Effectiveness and cost-efficiency of capture methods

The different capture methods were implemented a total of 1,859 times over 448 days, with the folding net being the most frequently employed method (n = 882), followed by the combination of egg and nestling culling (n = 365), the combination of folding net and net launcher (n = 301), net launcher on its own (n = 142), shooting (n = 92) and lastly egg culling on its own (n = 77) (Table 1).

The analysis of capture efficacies revealed that the capture method used, the period of the year and the interaction between these two variables significantly influenced capture efficacy (Appendix 6: Table A4). Efron's pseudo-R^2 coefficient for this model was 0.53. Pairwise comparisons using Sidak's test identified differences in the performance of the methods. During spring, the combination of egg and nestling culling was the most effective method, while folding net was the least effective. The rest of the methods performed similarly and lay in between in terms of capture efficacy. Outside of spring, the combination of folding net and net launcher was the most effective method, while folding net and net launcher, when used separately, were the least effective. The rest of the methods were not statistically significant from either the most or the least effective ones (Fig. 4A).

Results from the analysis of cost-efficiencies showed that the capture method used, the period of the year and the interaction between these two variables significantly influenced cost-efficiency (Appendix 6: Table A4). Efron's pseudo-R^2 coefficient for this model was 0.48. As with capture efficacies, there were methods that could not be exclusively assigned to one group. During spring, the combination of egg and nestling culling was the most cost-efficient method, while folding net

Table 1. Capture efficacy (number of parakeets captured per hour), cost-efficiency (cost per parakeet captured) and cost per hour of the capture methods used to manage the monk parakeet population in Madrid between 2021 and 2023. Sample sizes (n), mean values and standard deviation are shown for each capture method.

Season	Capture method	n	Birds/h	Cost/bird (€)	Cost/hour (€)
Spring	Shooting	22	1.4 ± 1.2	137 ± 76	192 ± 196
	Folding net	157	0.3 ± 0.5	212 ± 134	64 ± 113
	Net launcher	50	0.9 ± 0.8	76 ± 63	68 ± 83
	Egg culling	61	1.4 ± 1.6	215 ± 246	301 ± 487
	Folding net + net launcher	26	0.6 ± 0.5	83 ± 64	50 ± 56
	Egg + nestling culling	203	2.4 ± 1.7	134 ± 221	321 ± 577
Non-spring	Shooting	70	2.2 ± 1.5	102 ± 92	224 ± 254
	Folding net	725	1.2 ± 1.4	124 ± 148	149 ± 248
	Net launcher	92	1.2 ± 1.0	52 ± 37	62 ± 68
	Egg culling	16	0.8 ± 0.8	239 ± 233	191 ± 267
	Folding net + net launcher	275	1.7 ± 1.5	57 ± 86	97 ± 169
	Egg + nestling culling	162	2.1 ± 5.1	289 ± 235	607 ± 1554

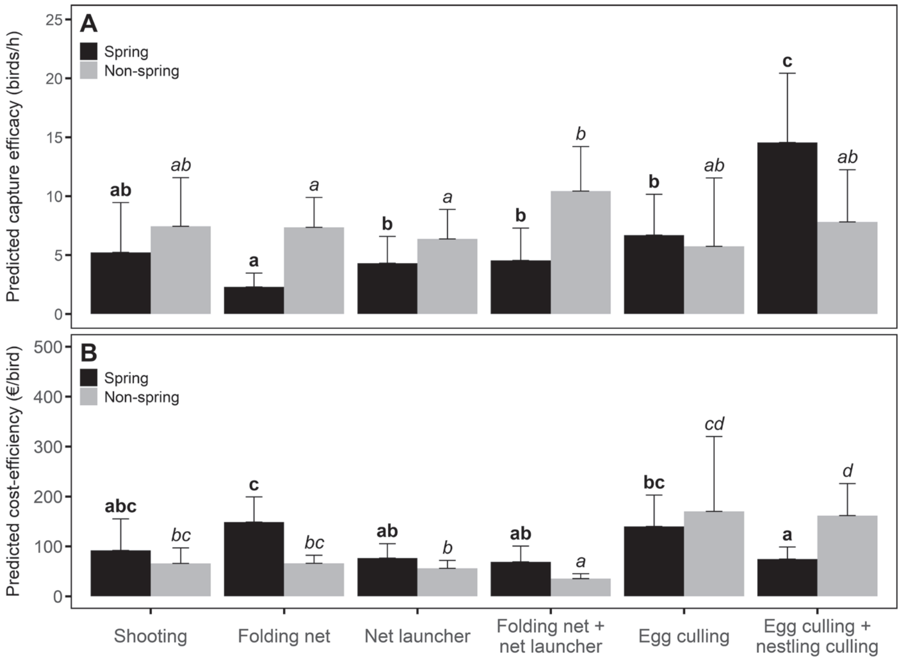

Figure 4. Results of the models for capture efficacies (**A**) and cost-efficiencies (**B**) across different capture methods. Bars represent least-square means, with 95% confidence intervals shown as error bars. Letters above the error bars indicate groups, based on Sidak's *post-hoc* test; bold letters denote spring values and italicised letters denote non-spring values. Methods without shared letters differ significantly, while those sharing at least one letter do not. Note that in panel **A**, higher capture efficacy indicates better performance, whereas in panel **B**, lower cost-efficiency values indicate better performance.

was the least cost-efficient. The remaining methods showed intermediate levels of cost-efficiency. Outside of spring, the combination of folding net and net launcher was the most cost-efficient method, while both aerial methods were the least cost-efficient. The rest of the methods performed similarly and lay in between in terms of cost-efficiency (Fig. 4B).

Table 2. Variation in population increase rate (r), based on different capture methods depending on the season. The model assumes an initial population size of 16,500 birds, an effort of 2000 hours annually and a period of control of 10 years.

Season	Capture method	R	Final population size (10 years)
Spring	Shooting	0.03	24,768
	Folding net	0.14	67,813
	Net launcher	0.10	46,237
	Egg culling	0.11	48,561
	Folding net + net launcher	0.13	57,449
	Egg culling + nestling culling	0.03	25,715
Non-spring	Shooting	-1.06	1,053
	Folding net	0.07	33,671
	Net launcher	0.07	33,975
	Egg culling	0.14	63,746
	Folding net + net launcher	-0.14	11,711
	Egg culling + nestling culling	0.06	33,014

Regarding the impact of individual capture methods on population growth rate, the models indicated that, during spring, shooting and the combination of egg and nestling culling were the most effective in reducing population growth rate. Outside of spring, however, shooting and the combination of folding net with net launcher were the most effective in reducing population growth rate (Table 2).

Decision-making models analysing the optimal combination of methods and effort levels revealed that, starting with a population of 10,000 individuals, the optimal strategy in spring was to allocate the maximum effort to both shooting and the combination of folding net and net launcher. This approach would eradicate the population within three years at a cost of €1,452,000. Outside of spring, the optimal strategy was to dedicate the maximum annual effort exclusively to the combination of folding net and net launcher, achieving eradication within three years at a cost of €582,000.

Food choice experiment

The different baits were tested a total of 30 times each in 27 different parks (Table 3). There were significant differences in the number of parakeets attracted by different types of bait (Appendix 6: Table A5). Efron's pseudo-R^2 coefficient for this model was 0.66. Pairwise comparisons revealed two groups of responses: one comprising the parrot feed and apple (Tukey's *post-hoc* test: $Z = -1.88$, $P = 0.24$) and the other one consisting of bread and the mixture of foods (Tukey's *post-hoc* test: $Z = 0.70$, $P = 0.90$). However, there were significant differences in the attractiveness between these two groups (Tukey's *post-hoc* test: $Z = -5.68$, $P < 0.001$), with bread and the mixture of foods attracting, on average, between two and six times more parakeets than the parrot feed and apple (Fig. 5A).

There were significant differences in the cost-efficiencies of attracting parakeets amongst different types of bait. Efron's pseudo-R^2 coefficient for this model was 0.73. Pairwise comparisons revealed that bread and the mixture of foods were the most cost-efficient baits, with no significant differences between them (Tukey's *post-hoc* test: $Z = 1.18$, $P = 0.64$). The apple bait was intermediate and the parrot feed was the least cost-efficient (Fig. 5B).

Table 3. Parakeets attracted per hour and cost-efficiency (cost per parakeet attracted) of the different baits used. Letter n represents the sample size and the rest of the values represent the mean, followed by the standard deviation.

Bait type	n	Parakeets attracted (birds/h)	Bait cost (€/kg)	Cost/bird (€)
Bread	30	27 ± 36	0.75	0.1 ± 0.2
Apple	30	13 ± 26	1.50	0.4 ± 0.3
Parrot feed	30	3 ± 8	2.05	0.8 ± 0.4
Mixture of the three baits	30	34 ± 44	1.43	0.2 ± 0.3

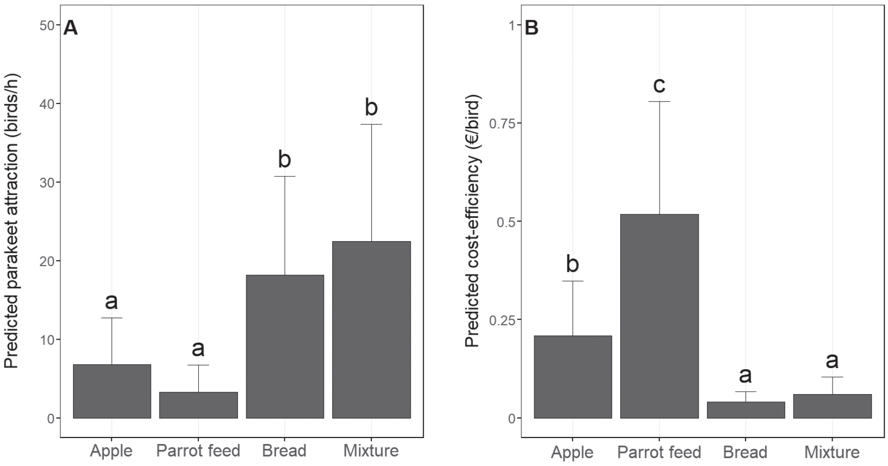

Figure 5. Results of the models for attraction efficacies (**A**) and cost-efficiencies (**B**) across different types of bait. Bars represent least-square means, with 95% confidence intervals shown as error bars. Letters above the error bars indicate groups, based on Tukey's *post-hoc* test. Methods without shared letters differ significantly, while those sharing at least one letter do not. Note that, in panel **A,** higher parakeet attraction indicates better performance, whereas, in panel **B,** lower cost-efficiency values indicate better performance.

Discussion

The monk parakeet management plan in Madrid, conducted from May 2021 to April 2023, aimed to minimise the city's population of this invasive species. As a result, the intervention reduced the projected total population (including nestlings, juveniles and adults) for 2023 and 2031 by approximately 50% compared to projections without management efforts. Notably, projections indicated that extending removal efforts at the intensity of the plan's second year for three additional years could have achieved near-total eradication of the species. The study showed that removing juveniles and adults was more than twice as effective in reducing population growth as removing eggs and nestlings, leading us to recommend prioritising the removal of juveniles and adults year-round rather than targeting eggs and nestlings. The plan employed various control methods, including shooting adults and juveniles perched on high branches, capturing them on the ground with folding nets and hand-held net launchers and culling eggs and nestlings. Capture efficacies and cost-efficiencies of these methods varied seasonally. In spring, shooting and the combination of egg and nestling culling were the most effective methods for reducing population growth rates; however, we do not recommend the latter due to evidence gathered against its use and its high associated costs. Outside spring, shooting and the combination of folding net and net launcher were most effective. When incorporating cost-efficiency into optimisation models, these indicated that, in spring, maximum effort should be allocated to both shooting and the combination of folding nets and net launchers. Outside of spring, the optimal strategy was to allocate maximum effort exclusively to the combination of folding nets and net launchers. These findings differ slightly from those derived using mixed models of capture efficacy and cost-efficiency, which are discussed later. Finally, two bait types – bread alone and a combination of bread, apple and parrot feed – were found to be most effective and cost-efficient for attracting parakeets to the ground.

The division into seasons allows for analysis of two biologically distinct periods for the monk parakeet: spring, when most of nesting occurs and the rest of the year, when reproductive activity decreases significantly (Senar et al. 2019). In spring, the method with the highest number of captures per hour was the combination of egg and nestling culling, which was expected given that most clutches occur during this season (Senar et al. 2019).

This high capture efficacy likely explains why, in our analyses, the combination of egg and nestling culling, along with shooting, were the two methods that most effectively reduced the population growth rate in spring. However, in parallel analyses, we concluded that culling eggs and nestlings is half as effective for population reduction as culling juveniles and adults. This, combined with several studies discrediting the effectiveness of egg culling, leads us to recommend against its use. Amongst these studies, some indicate that egg culling requires treating a large proportion of clutches to achieve a meaningful population impact (Coluccy et al. 2004; Baxter et al. 2010; Beston et al. 2016). Other research suggests that egg culling alone typically does not substantially reduce population size (Martínez-Abraín et al. 2004; Conroy and Senar 2009; Sanz-Aguilar et al. 2009; Esteban 2016), although it has proven effective for some species, such as the Canada goose *Branta canadiensis*, mute swans *Cygnus olor* and common ravens *Corvus corax* (Hindman et al. 2014; Beaumont et al. 2018; Brussee and Coates 2018). Additionally, parakeets may quickly detect culled eggs (whether oiled, punctured or replaced with dummies) and replace them, as observed in the U.K. (Animal and Plant Health Agency, unpublished data). Regarding nestling culling, no studies have assessed its effectiveness, likely due to welfare concerns and public opposition (Carrete et al. 2022). Nestling culling may be more effective than egg culling since nestlings have a higher probability of survival to reproductive maturity than unhatched eggs (Navarro et al. 1992; Ackerman et al. 2014). Furthermore, it may result in smaller replacement clutches or more frequent reproductive delays compared to egg culling, as seen in the red-backed shrike *Lanius collurio* (Antczak et al. 2009). Nevertheless, nestling culling is still less effective than methods targeting adults for several reasons: i) nestlings and juveniles have high natural mortality rates, meaning many would not survive to contribute to the population regardless of culling efforts (Bucher et al. 1991; Senar et al. 2019); ii) the method is restricted to a short window during the breeding season, whereas adult-focused methods can be applied year-round; and iii) synchronised reproduction requires significant, simultaneous effort, adding logistical challenges. These limitations reinforce our conclusion that egg and nestling culling is less effective and less practical than approaches focused on removing juveniles and adults.

Shooting was equally good as the combination of egg and nestling culling in reducing the population increase rate in spring and the best method in doing so outside of spring. This was expected because it has been the main control method deployed in most successful parakeet management programmes (Zaragoza, Spain: Esteban (2016); Balearic Islands, Spain: Orueta (2007), Molina et al. (2016); Uruguay: Bruggers et al. (1998), Linz et al. (2015); US: Neidermyer and Hickey (1977), Avery and Shiels (2017); but see the following for cases where shooting proved ineffective: Godoy (1963); Morgan and McNee (2000); Petersen and Grasso (2010)). Senar et al. (2021) estimated the capture efficacy and the cost of shooting for Zaragoza (Spain) and their results were much better than ours. However, there are factors with the shooting method in Madrid that could explain these discrepancies: unlike in Zaragoza, where shooting was allowed throughout the entire city, in Madrid, shooting was restricted to specific locations closed to the public. This may have limited the number of parakeets captured because shooting did not always take place in areas with high parakeet nest densities. Additionally, shooting was the only method with inactive periods, as it was decided to shoot at dawn and dusk when parakeet activity is highest in order to maximise captures, resulting in a period of inactivity between these shooting windows. These two factors may have led to an underestimation of the method's capture efficacy. Regarding cost-efficiency, the shooting team was the only subcontracted team, resulting in a proportionally higher salary compared to other teams hired by the company, thereby inflating the cost-efficiency estimates for this method. Additionally, it is important to note that, since there was only one shooting team, there is a high degree of collinearity between the team and the shooting method. This complicates the identification of the true predictor – whether it is the shooting method itself or the specific team – affecting the response variable (see Appendix 7). Despite these limitations, the shooting method performed quite well and we consider it a viable option for serious consideration both in spring and, particularly, outside of the spring season. Indeed, shooting could be particularly useful for targeting adults, the age class most likely to survive into the next breeding season, compared to eggs, nestlings and juveniles (Bucher et al. 1991; Conroy and Senar 2009). This is because adults seem less likely to fall into folding nets compared to juveniles, who are more naïve (Senar, pers. comm.). Therefore, shooting could be effective in targeting localised populations where adult birds no longer fall into capture nets, thereby facilitating subsequent nest removal with guarantees of not being rebuilt. Moreover, shooting can be an effective method for quickly eliminating incipient monk parakeet populations and preventing their dispersal and proliferation. Finally, we believe that shooting can also be considered one of the most ethical capture methods, as it is intended to cause immediate death to the animal and to avoid prolonged stress. However, for this method to be truly ethical and ensure immediate death, it must be carried out by expert shooters with animal welfare training.

Outside of spring, the second best method in reducing population increase rate was the combination of folding net and net launcher. The reason for the high effectiveness of the method is that, during summer, autumn and winter, parakeets descend to the ground to feed, relying on herbaceous vegetation and human-provided food, which facilitates their capture with nets. In contrast, during spring, parakeets have more food available in trees (e.g. new shoots and flowers) and do not need to come down to the ground (Postigo et al. 2021). Although there is not much precedent for these methods in managing monk parakeets, similar systems have been used successfully elsewhere (Florida: Avery et al. (2002); Barcelona: Senar et al. (2021)). While the folding net can go more unnoticed than other types of traps, repeated captures can make birds cautious, reducing long-term effectiveness. To prevent this, rotating capture sites regularly can help maintain effectiveness by keeping parakeets from learning patterns. Additionally, it is highly recommended to pre-bait the capture area with bread to attract parakeets to the area and potentially achieve more numerous captures.

The analysis of capture efficacy and cost-efficiency using mixed models aligns with the findings from the population growth rate reduction analysis and the optimisation model for method selection, with one notable exception: the shooting method. In the mixed model analysis, the efficacy of shooting appears to be underestimated, likely due to heteroscedasticity introduced by the lack of orthogonality in the control plan design. This limitation stems from the plan being tailored for technical rather than scientific purposes. However, when the random factor "team" is removed (Appendix 7), the mixed model results converge with those obtained from the demographic and optimisation models of Senar et al. (2021).

The food choice experiment was conducted to determine the most effective bait to use in order to increase the capture success of the folding net method. The baits that attracted the most parakeets were bread and the mixture bait composed of bread, apple and parrot feed. Our findings align with those of Postigo et al. (2021), who found that bread provided by humans ranked as the second most consumed food by monk parakeets in urban settings, just after grass (Poaceae family). Moreover, anthropogenic food has been identified as a key factor influencing the distribution of monk parakeets in Barcelona (Rodríguez-Pastor et al. 2012) and contributing to their breeding success and survival (Chamberlain et al. 2009). Since the bread and the mixture bait are equally cost-efficient, we recommend using only bread to simplify the food supply.

Conclusions and further prospects

This study evaluates the impact of the monk parakeet management plan implemented in Madrid from 2021 to 2023 on the population viability of the species. It also assesses the capture efficacy and cost-efficiency of different capture methods according to the season, concluding that the removal of juveniles and adults is more than twice as effective as the removal of eggs and nestlings in reducing population growth. For this reason, despite the superior capture efficacy of egg and nestling removal in spring, we recommend the use of the shooting method year-around, along with the combined use of the folding net and the net launcher outside of the spring season. The folding net allows for bulk captures, although it may have a bias towards naïve juveniles and requires pre-baiting to attract parakeets. In contrast, shooting is effective for targeting individuals, mainly adults, who have become wary of capture nets.

Additionally, the study tested different types of bait to determine the most effective option for attracting parakeets to the folding net, with bread being exclusively recommended.

It is also crucial to emphasise the importance of territorial coordination, as parakeets can easily move between neighbouring municipalities (Borray-Escalante et al. 2023). A lack of synchronised management across regions can lead to parakeets shifting to areas where previous control measures have created new, unoccupied niches, reducing the effectiveness of localised efforts. Therefore, a coordinated approach involving all affected municipalities is essential. This could include regular meetings to align strategies, share data and monitor parakeet movements across borders. Such collaboration can ensure a more comprehensive and effective control effort, minimising the risk of parakeets exploiting management gaps.

Additionally, management efforts must be sustained over time to prevent population recovery. Parakeets are highly adaptable and resilient birds, capable of quickly re-establishing populations if control measures are paused or halted. Continuous monitoring and control activities are essential to maintain the progress made in reducing parakeet numbers. This long-term commitment should involve regular assessments to adjust strategies based on updated data and research.

Sustainable funding and resources are also critical for maintaining ongoing efforts. Municipalities should seek to secure long-term financial support, possibly through partnerships with governmental and non-governmental organisations, to ensure that control measures can be sustained over time. Public education and community involvement are also crucial; raising awareness about the impact of invasive parakeets and encouraging public participation in reporting sightings can enhance the effectiveness of the management programme. Madrid's control plan included talks at academic institutions and for professionals in the field, along with the distribution of information leaflets. Looking ahead, we recommend also involving public administrators and educational institutions.

In summary, this study highlights the importance of rigorously testing management tools for invasive species, such as the monk parakeet, to ensure more effective and cost-efficient management of the species in the future. Coordinated, long-term efforts across municipalities are key to achieving lasting population reductions and ongoing collaboration between management teams and the scientific community is crucial for developing scientifically validated strategies.

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Additional information

Conflict of interest

Co-authors JB-G, FE and IA are employees contracted by MATINSA, the company awarded the contract by the Madrid City Council to control the monk parakeet population in Madrid from May 2021 to April 2023. One piece of information that we have omitted in the text due to financial considerations relates to how food was distributed in the feeders used to capture parakeets with folding nets. Knowledge gained from this trial-and-error process would have been beneficial for other companies in the event of a hypothetical future tender to continue controlling the parakeet population in Madrid.

Ethical statement

The monk parakeet (*Myiopsitta monachus*) is classified as an invasive exotic species in Spain under Royal Decree 630/2013, dated 2 August 2013. This Decree mandates that competent authorities control and manage the species in accordance with current animal welfare laws. The management carried out by MATINSA adhered to this regulation.

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Author contributions

All authors contributed to the conception and design of the study. The design, implementation and monitoring of the control plan were performed by FE, IA and JCO. Food choice experiment was performed by JB-G. Data analysis was performed by JB-G, IL-R and LC. The first draft of the manuscript was written by JB-G, IL-R and LC and all authors commented on other versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated during and/ or analysed during the current study are available from the corresponding author on reasonable request.

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Appendix 1

Public opposition

The management plan encountered public opposition at various stages, particularly at the beginning. This reaction was expected due to three main factors: the monk parakeet's perception as charismatic that many residents had adopted and normalised; legal requirements mandating the culling of captured individuals; and the high population density of Madrid, which increased the likelihood of public resistance.

The most notable public backlash occurred early on, when three individuals illegally entered a closed public park where shooting was underway. They filmed the operators and took a parakeet that had been shot, leading to widespread, often sensationalist, media coverage. In response, the plan prioritised using parks and green spaces that could be securely closed to prevent unauthorised access during shooting operations. These areas were mostly private, though certain public parks that met safety requirements were also utilised.

In addition to this widely-publicised event, operators occasionally faced verbal confrontations from the public while capturing parakeets using both ground and aerial methods and two minor protests occurred outside MATINSA's offices. These events, however, did not significantly disrupt the management plan. Importantly, strong support from the city council - especially in deploying police to de-escalate conflicts between operators and residents - was critical in maintaining operational continuity.

Appendix 2

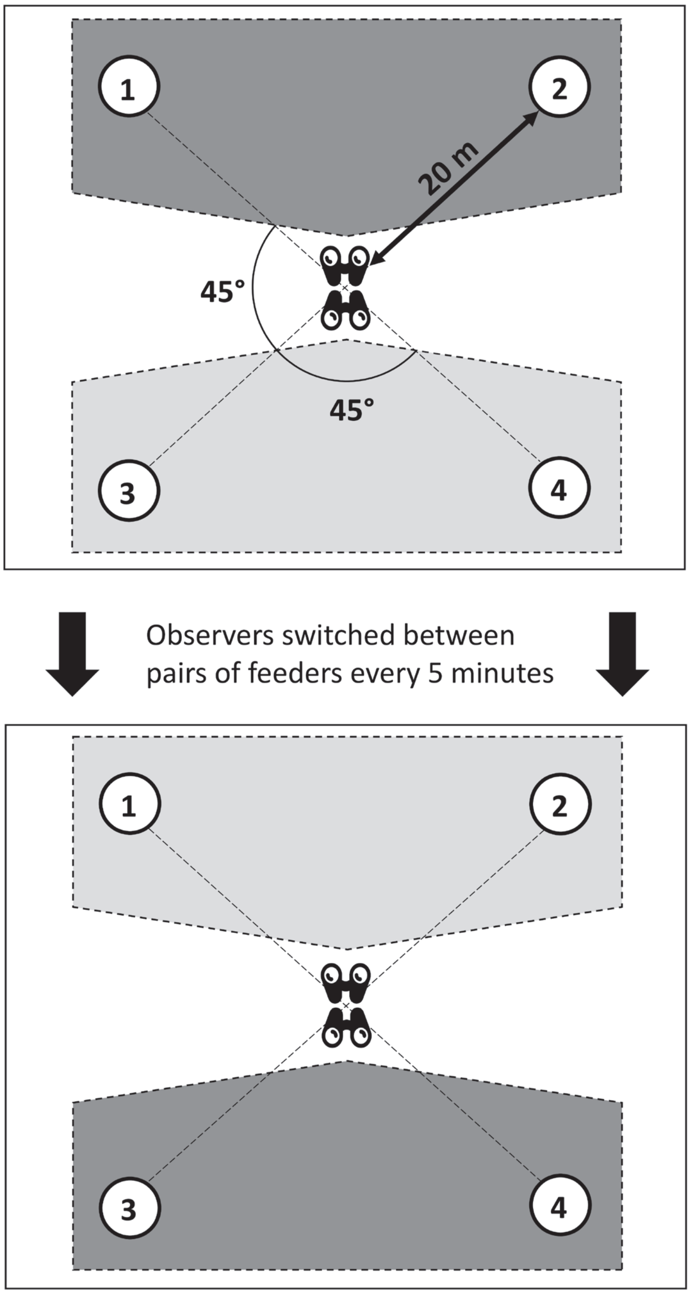

Figure A1. Diagram illustrating the positions of the two observers relative to the feeders, which are labelled with numbers from 1 to 4. Each feeder was placed approximately 20 m from the observers. Feeders observed by Observer 1 are shown in dark grey and those observed by Observer 2 are shown in pale grey.

Appendix 3

The population viability analysis was based on two transition matrices that modelled reproduction and survival rates across age classes. The first matrix contained the average values (Table A1), while the second included standard deviations to incorporate environmental stochasticity (Table A2). Most values were sourced from Senar (2021), with two exceptions: the nestling-to-juvenile survival probability, derived from Senar et al. (2019), and the uncertainty associated with survival rates, estimated by assuming a 10% standard deviation relative to the average. Reproduction rates indicate the number of nestlings produced annually by each individual based on age class; first-year juveniles were assigned a single clutch per year, while adults were assumed to produce two clutches annually.

Table A1. Average transition rates for the monk parakeet population in Madrid. The first row shows fecundity rates, expressed as the annual number of nestlings produced per individual in each age class. Subsequent rows represent age-class transition probabilities, indicating the likelihood of advancing to the next age class within a year.

Age class	Nestling	Juvenile 1st year	Adult 2nd year	Adult 3rd	Adult 4th	Adult 5th	Adult 6th	Adult 7th	Adult 8th	Adult +8th
Nestling	0	0.75	1.386	1.431	1.431	1.431	1.431	1.431	1.431	0
Juvenile 1st year	0.524	0	0	0	0	0	0	0	0	0
Adult 2nd year	0	0.61	0	0	0	0	0	0	0	0
Adult 3rd	0	0	0.81	0	0	0	0	0	0	0
Adult 4th	0	0	0	0.81	0	0	0	0	0	0
Adult 5th	0	0	0	0	0.81	0	0	0	0	0
Adult 6th	0	0	0	0	0	0.81	0	0	0	0
Adult 7th	0	0	0	0	0	0	0.81	0	0	0
Adult 8th	0	0	0	0	0	0	0	0.81	0	0
Adult +8th	0	0	0	0	0	0	0	0	0.81	0.81

Table A2. Standard deviations for transition rates in the monk parakeet population matrix, representing the variability incorporated into the population viability model to account for environmental stochasticity.

Age class	Nestling	Juvenile 1st year	Adult 2nd year	Adult 3rd	Adult 4th	Adult 5th	Adult 6th	Adult 7th	Adult 8th	Adult +8th
Nestling	0	0.32	0.456	0.475	0.475	0.475	0.475	0.475	0.475	0
Juvenile 1st year	0.0524	0	0	0	0	0	0	0	0	0
Adult 2nd year	0	0.061	0	0	0	0	0	0	0	0
Adult 3rd	0	0	0.081	0	0	0	0	0	0	0
Adult 4th	0	0	0	0.081	0	0	0	0	0	0
Adult 5th	0	0	0	0	0.081	0	0	0	0	0
Adult 6th	0	0	0	0	0	0.081	0	0	0	0
Adult 7th	0	0	0	0	0	0	0.081	0	0	0
Adult 8th	0	0	0	0	0	0	0	0.081	0	0
Adult +8th	0	0	0	0	0	0	0	0	0.081	0.081

Appendix 4

Table A3. Total and monthly removal of monk parakeets by age class from May 2021 to April 2023. Parakeets are categorized into three age classes: adults and juveniles, nestlings, and eggs. Data from 2021 cover the months of May to December, data from 2022 cover January to December, and data from 2023 cover January to April.

		2021								2022											2023			
	Total	**M**	**J**	**J**	**A**	**S**	**O**	**N**	**D**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**	**D**	**J**	**F**	**M**	**A**
Adults + juveniles	14,321	2	0	9	267	466	320	591	657	941	409	276	210	342	555	522	1,07	1,260	1,674	959	1,166	1,241	767	24
Nestlings	2,822	178	18	9	9	1	0	0	0	0	0	277	1,569	571	103	61	23	3	0	0	0	0	0	0
Eggs	2,062	19	37	6	15	13	1	1	0	0	92	884	471	330	93	33	32	4	0	0	0	0	16	15

Appendix 5

Time dedication to each capture method

During the control plan, up to 10 teams were working simultaneously to reduce the monk parakeet population. Nine teams were consistently composed of two people (hereafter referred to as “standard teams”), while one team consisted of 1 or 2 people depending on the capture method and the day (hereafter referred to as the “shooting team”). This last team was the only one authorised to use an air rifle. Each worker’s workday lasted 8 hours. To standardise calculations for both the time dedicated to capture methods and the associated costs, the workday for teams of two was set at 16 hours. An exception was made for the shooting team: when using the folding net or net launcher, only one person was involved, so the workday was 8 hours. On days when air rifle shooting was employed, either one or two people were involved, depending on the expected number of parakeets, so the time dedicated to air rifle shooting was standardised to 12 hours – the average of 8 hours (one person) and 16 hours (two people).

Additionally, two auxiliary activities were conducted during the management plan that, although not capture methods per se, had to be factored into calculations because they were often combined with parakeet capture: nest removal and baiting of feeders. In the case of nest removal, the time dedicated to this activity was subtracted from the corresponding workday to accurately assess the time dedicated to capturing parakeets. Regarding baiting of feeders, we categorised this activity in two ways: “average baiting”, which is the average time taken to bait an area before the capture day and which was always added to the associated folding net activity; and “day baiting”, which refers to daily baiting carried out by the teams to maintain an effective rotation of capture sites. The time spent on "day baiting" was subtracted from the workday, while it was added to all instances of capture with the folding net. Additionally, on days when nest removal or "day baiting" occurred, the proportional travel time associated with these two auxiliary activities was also subtracted from the workday.

Below, we outline how we calculated the time dedicated to each capture method, depending on the team performing it and any other methods being used simultaneously. We will separate the explanations for the shooting team and the standard team, as well as by the number of methods used per day. The time for average baiting is not specified because it varied for each of the 10 teams. Combinations not explained did not occur.

Shooting team

One method per day

- Shooting: 12 h.
- Folding net: 8 h + average baiting.
- Net launcher: 8 h.

Two methods per day

- Shooting and day baiting: 12 h – 2 h (day baiting) – 1 h (commute).
- Folding net and day baiting: 8 h – 1.25 h (day baiting) – 40 min (commute) + average baiting.

Standard teams

One method per day

- Folding net: 16 h + average baiting × 2.
- Net launcher: 16 h.
- Egg culling: 16 h.

Two methods per day

- Folding net and day baiting: 16 h – 3 h × 2 (day baiting) – 1 h 20 min (commute) + average baiting × 2.
- Folding net and net launcher: 16 h + average baiting × 2.
- Net launcher and pre-baiting: 16 h – 3 h × 2 (day baiting) – 1 h 20 min (commute).
- Egg culling and nestling culling: 16 h.
- Egg culling and nest removal: 16 h – (1 h 20 min × n° of nests removed) – 1 h 20 min (commute).

Three methods per day

- Folding net and net launcher and day baiting: 16 h – 3 h × 2 (day baiting) – 53 min (commute) + average baiting × 2.
- Egg culling and nestling culling and nest removal: 16 h – (1 h 20 min × n° of nests removed) – 53 min (commute).

Cost per capture method

After calculating the daily time dedicated to each capture method, we determined the cost of implementing these methods in order to subsequently calculate cost-efficiency values. The following associated costs were considered:

Salaries

- Shooting team: 92.6 € / h × day.
- Standard teams: 11.9 € / h × person.
- Manager: 12.5 € / h × person.
- Veterinarian: 32 € / h × person.

Vehicle rental

- Aerial work platform: 79 € / h.
- Light vehicle: 8.5 € / h.
- Gasoline: 5 € / car × day.

Other Costs

- Bait: 4.7 € / baiting event.
- Clinical equipment: 49 € / any capture method used × day.
- Consumables: 12.7 € / any capture method used × day.
- Incineration of carcasses: 4.7 € / any capture method used × day.
- Signals and markings: 27.7 € / any capture method used × day.

Price calculation

Shooting team

- Shooting: salary × h + incineration + veterinarian × 0.7.
- Folding net: salary × h + clinical equipment + consumables + incineration + veterinarian.
- Net launcher: salary × h + clinical equipment + consumables + incineration + veterinarian.
- Folding net and net launcher: salary × h + clinical equipment + consumables + incineration + veterinarian.

Standard teams

- Folding net: salary × h + bait + clinical equipment + consumables + incineration + veterinarian + gasoline.
- Net launcher: salary × h + clinical equipment + consumables + incineration + veterinarian + gasoline.
- Folding net and net launcher: salary × h + bait + clinical equipment + consumables + incineration + veterinarian + gasoline.
- Egg culling: salary × h + aerial work platform × h + light vehicle × h + signals and markings + gasoline.
- Egg culling and nestling culling: salary × h + aerial work platform × h + light vehicle × h + signals and markings + clinical equipment + consumables + incineration + veterinarian + gasoline.

Appendix 6

Table A4. AIC and Efron's pseudo-R^2 values for the selection of the best model for capture efficacy and cost-efficiency of the capture methods. Following the order of the best model selection process, the selection of random factors is presented first, where a full model is fitted and different random structures are compared. Once the random structure is chosen, the selection of fixed effects is performed, where different fixed structures are compared while keeping the previously-selected random structure fixed. The best model (lowest AIC) is indicated in boldface type.

	Capture efficacy		**Cost-efficiency**	
Random factors selection	**AIC**	**Efron's pseudo-R^2**	**AIC**	**Efron's pseudo-R^2**
No random factors	11906	0.16	5526	0.19
(1 | date)	11879	0.28	5519	0.27
ou[1] (time[2] + 0 | season)	11845	0.26	5488	0.26
ou (time + 0 | covariate[3])	11843	0.26	5487	0.27
(1 | team)	11817	0.21	5453	0.24
ou (time + 0 | method)	11792	0.33	5444	0.36
(1 | team) + (1 | date)	11777	0.37	5441	0.33
(1 | team) + ou (time + 0 | season)	11739	0.35	5414	0.31
(1 | team) + ou (time + 0 | covariate)	11737	0.35	5413	0.32
ou (time + 0 | team)	11710	0.44	5375	0.44
(1 | team) + ou (time + 0 | method)	11694	0.41	5370	0.40
(1 | team) + ou (time + 0 | team)	**11684**	**0.50**	**5368**	**0.47**
Fixed factors selection				
Null model	11781	0.481	5454	0.429
Season	11781	0.48	5450	0.427
Method	11714	0.456	5391	0.443
Method: Season	11684	0.499	5368	0.471
Method: Season + method	11684	0.499	5368	0.471
Method: Season + season	11684	0.499	5368	0.471
Method*Season	**11684**	**0.499**	**5368**	**0.471**

[1] The Ornstein-Uhlenbeck (OU) covariance structure models temporal autocorrelation.
[2] Numerical factor used to model temporal autocorrelation.
[3] Factor with a single level, used to specify that the temporal autocorrelation applies to all observations.

Table A5. AIC and Efron's pseudo-R^2 values for the selection of the best model for attraction efficacy and cost-efficiency of the bait types. See Table A4 for more information on the best model selection process. The best model (lowest AIC) is indicated in boldface type.

	Attraction efficacy		**Cost-efficiency**	
Random factors selection	**AIC**	**Efron's pseudo-R^2**	**AIC**	**Efron's pseudo-R^2**
No random factors	963	0.171	476	0.354
(1 | park) + (1 | date)	961	0.496	476	0.538
(1 | date)	959	0.496	473	0.538
(1 | park)	**959**	**0.496**	**473**	**0.538**
Fixed factors selection				
Null model	985	0.237	520	0.246
Bait type	**951**	**0.495**	**468**	**0.538**

Appendix 7

Due to the high collinearity between the shooting method and the team responsible for conducting the shooting (correlation coefficient of 0.86), it is unclear which of these factors is influencing the response variable. To assess whether the "team" factor was masking the performance of the shooting method, we adjusted the models for capture efficacy and cost-efficiency without including the team factor as random factor. In this way, it can be observed that the capture efficacy of the shooting method shows a significant increase both during and outside of spring (Fig. A2B), being amongst the most effective methods in both seasons, compared to the model including the team factor (Fig. A2A). Regarding cost-efficiency, an improvement can be observed in spring when the team factor is not included (Fig. A3B). However, the mixed model selection, based on AIC and Efron's pseudo-R^2, indicated that the team factor should be included in the model. Therefore, we have included the model with the team factor in the main text and placed the model without it in the Appendices.

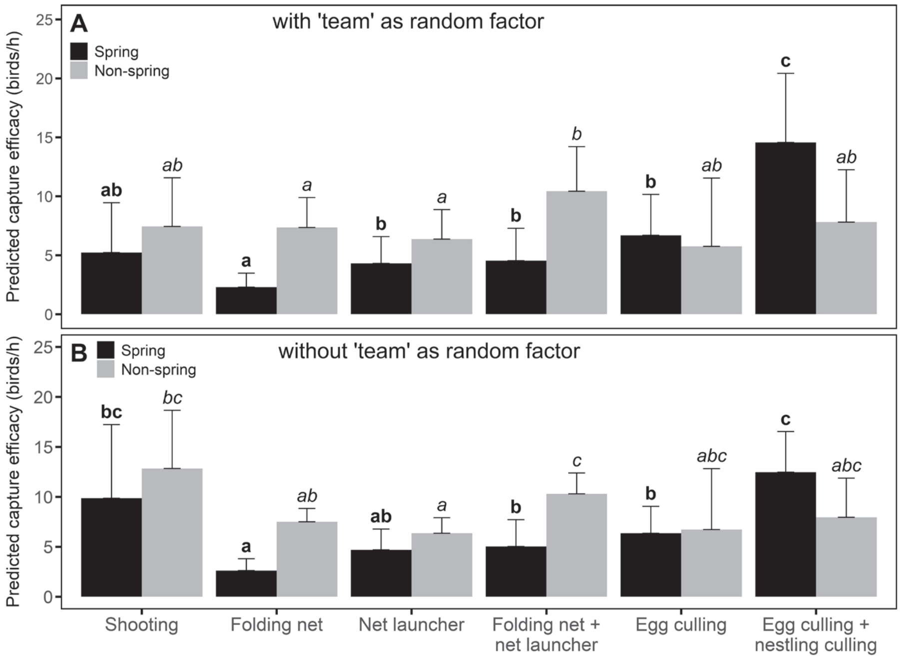

Figure A2. Results of the models for capture efficacies when the "team" factor is included as random factor (**A**) and when it is not (**B**). Bars represent least-square means, with 95% confidence intervals shown as error bars. Letters above the error bars indicate groups based on Sidak's *post-hoc* test; bold letters denote spring values and italicised letters denote non-spring values. Methods without shared letters differ significantly, while those sharing at least one letter do not. Note that a method performs better, the higher its capture efficacy.

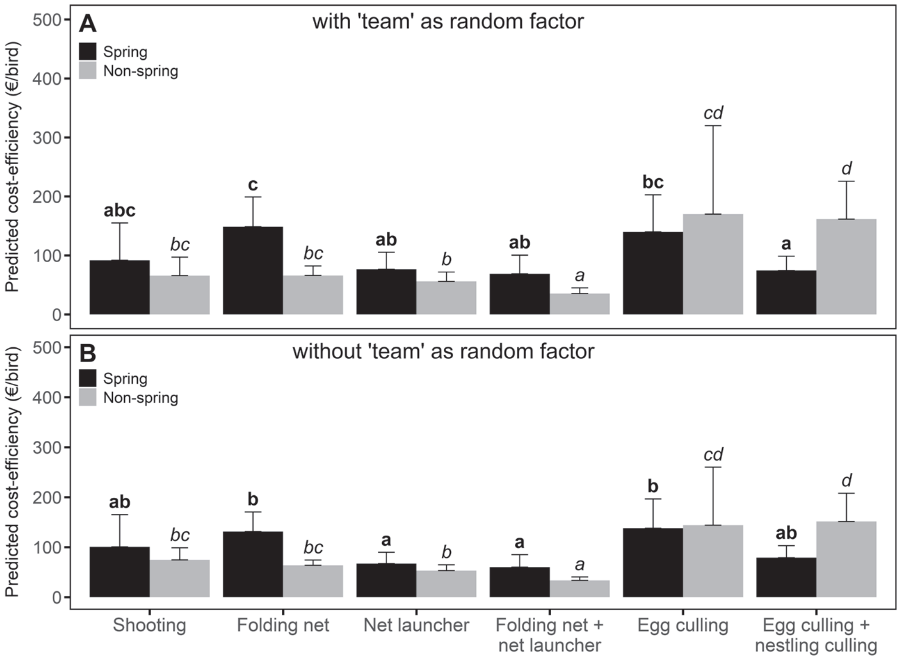

Figure A3. Results of the models for cost-efficiencies when the "team" factor is included as random factor (**A**) and when it is not (**B**). Bars represent least-square means, with 95% confidence intervals shown as error bars. Letters above the error bars indicate groups based on Sidak's *post-hoc* test; bold letters denote spring values and italicised letters denote non-spring values. Methods without shared letters differ significantly, while those sharing at least one letter do not. Note that a method performs better, the lower its cost-efficiency.

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Research Article

Status assessment of non-native terrestrial species in Antarctica

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Abstract

Antarctica has been subject to direct human activity for a little over 200 years. In recent decades, the combination of sharp increases in human activity and regional climate change, particularly around the Antarctic Peninsula and Scotia Arc, have placed the terrestrial and freshwater environment under increased threat of non-native species introduction and establishment. Policymakers, including those on the Antarctic Treaty Consultative Meeting's Committee for Environmental Protection, need accurate and up-to-date information on the presence and status of non-native species within Antarctica upon which to base their decision-making. Here we collate available information to consider the status of known non-native species in the terrestrial Antarctic, and how this has changed in the past decade. Of known establishments, we found 46% to have been deliberately introduced during historical transplant experiments and subsequently removed, 36% were non-experimental introductions, and 18% only survive(d) synanthropically (i.e., associated with Antarctic facilities). All non-native species currently established in the natural Antarctic environment are located in either the Antarctic Peninsula, South Shetland Islands or South Orkney Islands (i.e., the maritime Antarctic region, with none in the continental Antarctic), with invertebrate species dominating. Most of the currently established non-native species have now been present for more than a decade, though the more recent appearance of non-native flies in station sewage treatment plants and their expansion into the Antarctic environment is a major cause for concern. While there has been some success in eradicating introduced plants, management of introduced invertebrates in the natural environment has largely not been attempted. Considerable scope exists for the Antarctic Treaty Parties to better coordinate non-native species management across the invasion continuum.

Key words: Alien, biological invasion, biosecurity, Committee for Environmental Protection, environmental management, risk

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Introduction

Humans first arrived in Antarctica in the 1820s and, in the process of travelling to the region, almost certainly brought the first non-native species with them (Headland 2009). Since that time, and likely correlated with the level of human activity, the number and diversity of introduced non-native species in the region has grown (Frenot et al. 2005; Hughes et al. 2015). For the purposes of this study, a non-native species is defined as a species existing beyond its natural dispersal range, transported either deliberately or unintentionally by human activity, and an invasive species is a non-native species that expands its distribution following establishment in a newly colonised area and has a negative impact upon native species and/or ecosystem function (Blackburn et al. 2011; Antarctic Treaty Secretariat 2019). The progressive extension of national Antarctic programme footprints across the continent, including the construction of new research stations, plus the expansion of the tourism industry, particularly in the Antarctic Peninsula region, is likely to have increased opportunities for species introductions, as well as for the intra-regional transfer of species native to different regions in Antarctica (also termed as 'native-alien populations', see Nelufule et al. 2022) (Pertierra et al. 2017a; Brooks et al. 2019; Hughes et al. 2019). For much of Antarctica the extreme environmental conditions and scale of geographical isolation, compared to many other parts of the planet, have provided a barrier to establishment of non-native species (González-Herrero et al. 2024). However, climate change has already ameliorated environmental conditions in the Antarctic Peninsula region (which has warmed by c. 3 °C since the mid-20th Century) and the warming now detected across other areas of the continent is predicted to accelerate (Bracegirdle et al. 2019; Turner et al. 2019; Clem et al. 2020). Under these rapidly changing circumstances the risk of species introduction, establishment and subsequent transition to invasive status is a major cause for concern (Convey and Peck 2019; Duffy and Lee 2019; Lee et al. 2022a).

The Committee for Environmental Protection (CEP), created under the terms of the Protocol on Environmental Protection to the Antarctic Treaty (also known as the Environmental Protocol or Madrid Protocol), is the body formally tasked with the provision of advice to the Antarctic Treaty Consultative Meeting (ATCM) on environmental issues concerning the Antarctic Treaty area (i.e., all land, sea and ice areas south of latitude 60°S), as well as relating to dependant and associated ecosystems north of that latitude (Sánchez and McIvor 2007). The remit of the CEP includes non-native species issues, with policy advances including the development of the CEP Non-native Species Manual, which was first drafted in 2011 and underwent a major revision in 2016 (Antarctic Treaty Secretariat 2019), and the inclusion of non-native species in the CEP Climate Change Response Work Programme (Antarctic Treaty Secretariat 2024a) and CEP Five-year Work Plan (Antarctic Treaty Secretariat 2024b). To build on this work, policymakers rely upon accurate, apolitical and up-to-date information upon which to base their decision-making. A challenge for researchers is how best to provide this information (McIvor 2020; Hughes et al. 2022, 2023)

A number of studies have attempted to consolidate records of mostly terrestrial and freshwater non-native species in the Antarctic Treaty area, with some also extending to the sub- and wider peri-Antarctic islands. Few putative records of introductions of non-native marine species exist in the Antarctic Treaty area, with virtually no confirmed instances of establishment in either the short or the longer term (McCarthy et al. 2019). Smith (1996) provided a chronological account of all known experimental and accidental introductions of higher plants to the Antarctic Treaty area and discussed the associated impacts and conservation issues. Twenty years ago, Frenot et al. (2005), in their influential synthesis, recorded instances of plant, vertebrate, invertebrate, microbial and marine species introductions across the wider Antarctic region (i.e., also including the core sub-Antarctic islands) and placed these into the context of a rapidly changing environment. In 2012, Greenslade and co-authors produced two papers describing the non-native collembolan fauna of Deception Island (South Shetland Islands) and the sub-Antarctic (Greenslade and Convey 2012; Greenslade et al. 2012). Three years later, in 2015, and following the submission of regular updates on non-native species introductions to the CEP, Hughes et al. (2015) produced a list of species thought to be established in Antarctica at that time. Most recently, Leihy et al. (2023) produced a dataset detailing species known or inferred in the literature to have been introduced to the terrestrial and freshwater environments of Antarctica and the sub-Antarctic as well as to some lower latitude peri-Antarctic islands, although most of these records represent the latter island regions rather than the Antarctic Treaty area and do not include any explicit assessment of evidence of establishment.

In this study, we revise, update and expand the dataset of Leihy et al. (2023), focusing only on the terrestrial environment within the Antarctic Treaty area. We added 125 additional records of established non-native species and applied strict criteria to existing records to identify only established species (as distinct from those introduced but that did not establish, or that are now known to be native) which led to the removal of 103 records. We also included in our dataset additional information pertaining to the records, including geographical coordinates. We used this dataset to investigate trends and patterns in non-native species introductions and survival and assessed whether there have been any major status changes in the last decade (i.e., since the publication of Hughes et al. (2015). We intend this information to be of use to researchers, policymakers and environmental practitioners engaged in policy development and management of non-native species within the Antarctic Treaty area and beyond.

Methods

Collation of data for the database

All non-native terrestrial and freshwater species records located within the Antarctic Treaty area were within the scope of our study. Microbial introductions, except for a limited range of fungal species, were not included to any substantial degree (but see Cowan et al. 2011 and Hughes et al. 2018). As a starting point, a recently published list of introduced and invasive non-native species present in the broader Antarctic region was obtained from Leihy et al. (2023). The 218 records relating to the Antarctic Treaty area in the dataset of Leihy et al. (2023) were considered to be within the study's scope and were reassessed using a strict set of criteria to robustly identify species records that could with confidence be considered to have been anthropogenically introduced and subsequently established in Antarctica.

Records were excluded when: (i) the species is known to be native to the area of introduction; (ii) there is no evidence of establishment in Antarctica, either in the natural environment or in human-associated locations such as station buildings (e.g., the species was immediately removed or destroyed upon introduction, or there was no evidence the species had survived *in situ* and/or may have arrived in Antarctica already dead); (iii) the evidence from the source reference was too unreliable or weak for it to be included (e.g., there was no clear evidence of the introduction being human-mediated, or there was potential mis-identification); (iv) the species were vertebrates that were deliberately introduced for indoor experimental purposes (e.g. hamsters (Stewart 1990)), as pets (e.g., rabbits and cats), for food (e.g., pigs, sheep, cows), or as working animals (e.g., horses, dogs); (v) the record was of experiments where plants were cultivated indoors; (vi) the record was for plant propagules, but without the presence of developing or mature plants; or (vii) duplicate records. Application of these criteria resulted in 103 records being rejected from the published list of Leihy et al. (2023). The remaining 115 records were then supplemented with a further 125 additional records that satisfied the criteria which were present in older primary literature or had been recently published (literature published up until May 2024 was considered). Records were identified in publications through a systematic search on Google Scholar (https://scholar.google.com/) using the search terms 'Antarctic*' and 'non-native' or 'alien' or 'invasive'. However, in almost all cases, the additional records were identified based on the bibliographies of the authors who collectively have undertaken research on Antarctic non-native species for more than 60 years. In total, the new dataset contains 240 records of non-native species establishment events in the Antarctic Treaty area. Where possible, citations were made to the original source literature, rather than to existing literature reviews, compilations or lists of Antarctic non-native species (e.g., those of Pugh 1993, 1994; Smith 1996; Headland 2012; Hughes et al. 2015). The current dataset includes species survival time (see definition below) and location coordinates for the records. The original sources were checked for each record and information was confirmed and/or further details were added. An explanation and details of the field names used in the database are provided in Table 1. Field names are consistent with Darwin Core Standards where possible (see: https://dwc.tdwg.org/; Wieczorek et al. 2012). Details of the full scientific names, including the kingdom, phylum, class, order, family and species, were based on information contained within the Global Biodiversity Information Facility (https://www.gbif.org/). The dataset and metadata for this manuscript are freely available from the UK Polar Data Centre (https://doi.org/10.5285/afeb9f5e-bd69-4e3d-9d50-e935134f4c78). The dataset was also made available to the Committee for Environmental Protection in May 2024 as an interactive online application (SCAR 2024; https://saer-non-nativespecies.data.bas.ac.uk).

The distance from each record to the nearest national operator facility and visitor landing site was computed using the COMNAP facilities database and International Association of Antarctica Tour Operators (IAATO) landing site data (available from: https://iaato.org/information-resources/data-statistics/), respectively, and the 'Near' tool in ArcGIS Pro (v3.2). As a proxy for survey effort and biological knowledge in the vicinity of the site of establishment, the average distance to the ten closest biodiversity occurrence records (i.e., records of native Antarctic species with location and observation time) was computed using the recently available ice-free terrestrial biodiversity database (Terauds et al. 2025) and the 'Generate near table' tool in ArcGIS Pro (v 3.2). R version 4.2.2 was used for data visualisation (R Core Team 2022).

Results

Spatial distribution and means of introduction

In total we identified reports of 112 non-native species that have established at some point in time at 67 sites across Antarctica (Fig. 1; Table 2). These species can be divided into three main categories, representing their means of introduction, i.e., deliberately, unintentionally and present in the natural environment, or unintentionally and present inside buildings (here termed synanthropic).

Table 1. Explanation and details of the field names used in the Antarctic Treaty area non-native terrestrial species database.

Field name	Notes
record ID	Unique identifier for each record in database
occurrence status	The species is 'present' or 'absent' within the Antarctic Treaty area, or occurrence 'uncertain'.
category of introduction	The category describes how the introduction occurred, i.e., 'deliberate experiment' (introduced for experimental purposes at the given location), 'non-experimental introduction' (unintentional introduction to the Antarctic natural environment), or 'synanthropic' (species that have been introduced to and colonised human infrastructure within the Antarctic Treaty area).
scientific name	Scientific name of the species, e.g. *Poa annua*
Authorship	The authorship of the species/taxon name, e.g. 'L.' or 'Baker, 1965'
vernacular name	Common name, e.g., annual bluegrass.
kingdom	Biological classification
phylum	Biological classification.
class	Biological classification.
order	Biological classification.
family	Biological classification.
decimalLatitude and decimalLongitude	Coordinates for each of the records were obtained using, in order of priority, (i) the original source (where many newer citations provided the exact coordinates, or where older citations provided a detailed map that could be used to determine the coordinates using Google Earth), (ii) in the case of the site being scientific infrastructure, the Council of Managers of National Antarctic Programs (COMNAP) Antarctic facilities database (https://www.comnap.aq/antarctic-facilities-information), or (iii) in the case of the site being within or close to an Antarctic Specially Protected Area (ASPA), the ASPA management plans which were available from the Antarctic Treaty Secretariat Antarctic Protected Area database (https://www.ats.aq/devph/en/apa-database), or (iv) the placename coordinates detailed in the SCAR Composite Gazetteer of Antarctica.
Location	Named location of the record as given in the reference/s.
ACBR_ID	Antarctic Conservation Biogeographic Region (ACBR; see Terauds and Lee 2016) in which the species was located.
ASPA_ID	Further information was provided on which (if any) Antarctic Specially Protected Area (ASPA) the record occurred within.
establishment means	The mechanism of introduction and/or details of the source population.
pathway	The process by which the species came to be in the given location, e.g., research or transportation of habitat material.
first observation year	The year the species was first observed at the recorded location within the Antarctic Treaty area.
eradicationStatus	Status of whether or not the species has been eradicated, and if relevant, noting if the species was removed upon conclusion of a scientific experiment, died out without human intervention, is subject to on-going eradication efforts, or is still currently present.
eradication year	The year the species was eradicated, removed, or died out (if applicable; see 'eradicationStatus').
survival time	The period the species remained viable within the Antarctic Treaty area before either dying out or being removed, which is important for identifying and understanding the most persistent biological groups and the pace of management action. The survival time was calculated as the number of years or months (as relevant) between the first observation of the species at the given location in Antarctica and January of 2024.
occurrence remarks	Including, as relevant, details of the source population, abundance, etc.
first publish year	The year the record was first published in the academic literature (as available).
references	Original references (where available) and associated references relating to the species introduction to the Antarctic Treaty area in abbreviated form. Full references are available in the 'references' csv.
Leihy_record ID	The record identification number used in Leihy et al. (2023) (where available).
CoL_Taxon ID	Catalogue of Life (https://www.catalogueoflife.org/) species identifier.

Table 2. Number of records and species allocated to each introduction category group.

No.	Group	No. of records	No. of species
1	Species introduced deliberately to the natural Antarctic environment during transplantation experiments (and then removed)	112	67[1]
2	Non-experimental introductions of non-native species to the Antarctic natural environment	87	27[2]
3	Non-experimental introductions of non-native species persisting synanthropically	41	22[3]
	Total	240	112[4]

[1] Excludes three records that had insufficient taxonomic information.
[2] Excludes five records that had insufficient taxonomic information.
[3] Excludes four records that had insufficient taxonomic information.
[4] Some species are common to more than one group.

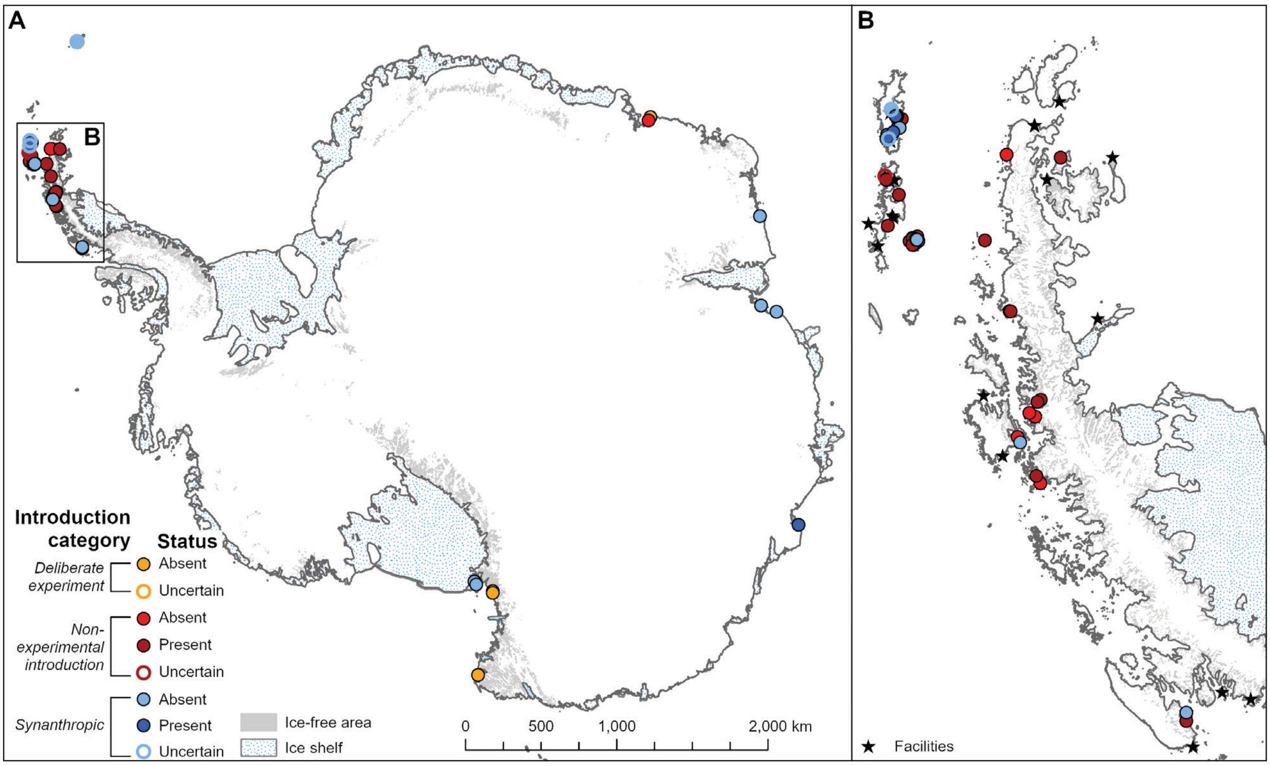

Figure 1. Distribution of non-native species records across **A** the entire Antarctic continent and **B** the northern Antarctic Peninsula. Map **B** is an inset of Map **A** and the legend in Map **A** is also relevant to Map **B**. Records are coloured by introduction category and occurrence status.

(i) Species introduced deliberately to the natural Antarctic environment during transplantation experiments

The largest group of records comprises 112 reports of at least 67 plant species (this number excludes records that had insufficient taxonomic information to identify the record to species level) that were introduced deliberately to Antarctica during transplantation experiments. Acknowledging the lack of available information for some records, as far as we can ascertain the great majority if not all deliberate introductions to Antarctica have been 'experimental introductions' (transplantation experiments) that were conducted at various times during the 20th Century. Transplantation experiments within the Treaty area involving species from outside the Antarctic would still be permitted through Annex II to the Protocol (but only under strict conditions intended to minimise the risk of long-term environmental impact). However, we are not aware of any such experiments having been undertaken in recent years (but see Câmara et al. (2021)). Most experimental species introductions were undertaken on Signy Island (South Orkney Islands), involving plants originating from the Scottish mountains (the Cairngorms), the Falkland Islands and South Georgia (Edwards and Greene 1973; Edwards 1980). However, a smaller number of reports are available from Port Lockroy (Goudier Island), Cierva Point (Danco Coast) and from the continental Antarctic sites of Cape Hallett, Granite Harbour and Syowa Station involving plants originating from, e.g., South America, Japan, the UK, or other parts of Antarctica (intra-regional transfer) (Corte 1961; Holdgate 1964; Young 1970; Smith 1996). There is no indication that any of these plant introductions remain *in situ* today, with all either dying during, or being removed at the termination of, the experiments.

(ii) Non-experimental introductions of non-native species to the Antarctic natural environment

Eighty-seven records of at least 27 species were introduced as a result of non-experimental activities. Established species in continental Antarctica were limited to only two locations, the grass *Puccinellia tenella* located very close to a refuge building c. 25 km from Syowa Station, Enderby Land, and five plant species (*Stellaria media*, *Rumex pulcher*, *Puccinellia distans*, *Oxybasis rubra* and *Alopecurus geniculatus*) at Progress II Station, Larsemann Hills; none of which remain (Russian Federation 1999; Tsujimoto et al. 2010). Records for currently surviving species are located on the western Antarctic Peninsula, the South Shetland Islands and South Orkney Islands (all within the maritime Antarctic). All but two of these records relate to invertebrate species, with the majority being Collembola or Acari at sites of a regular national Antarctic operator or tourism industry activity (e.g., see Russell et al. 2013). The two plant records relate to the presence of the invasive grass *Poa annua* in the immediate vicinity of Arctowski Station (King George Island) and the subsequent dispersal of this grass into the nearby Antarctic Specially Protected Area (ASPA) 128 Western Shores of Admiralty Bay, King George Island, South Shetland Islands (Galera et al. 2017, 2019, 2021). Reports of insects (Diptera) include *Trichocera maculipennis* on King George Island and *Eretmoptera murphyi* on Signy Island (South Orkney Islands) (Burn 1982; Hughes and Worland 2010; Volonterio et al. 2013; Potocka and Krzeminska 2018; Bartlett et al. 2020; Remedios-De León et al. 2021). The only annelid is the enchytraeid worm *Christensenidrilus blocki*, that was likely introduced to Signy Island in the same plant transplant experiment that led to the establishment of *E. murphyi* (Block and Christensen 1985).

Several non-native plant species that established in the Antarctic natural environment have been eradicated. Other than its large established population in the vicinity of Arctowski Station, King George Island, *P. annua* has been detected and eradicated, as single or small numbers of individual plants, at various locations across the Antarctic Peninsula, South Shetland Islands and South Orkney Islands (Molina-Montenegro et al. 2012, 2014; Malfasi et al. 2020), while its congener *P. pratensis* was eradicated from close to ASPA 134 Cierva Point, Danco Coast, Antarctic Peninsula in 2015 (Corte 1961; Pertierra et al. 2013, 2017b). Plants have also been eradicated from East Antarctic locations, including the five species from Progress II Station and *Pu. tenella* at a site near Syowa Station, mentioned earlier. *Nassauvia magellanica* and *Gamochaeta nivalis* were first reported from Whalers Bay in 2010 and subsequently respectively eradicated or washed away, but it was not clear if they had colonised by natural or anthropogenic means (Smith and Richardson 2011). This illustrates an important and unresolved challenge for authorities to assess when new species are discovered in the natural Antarctic environment (Hughes and Convey 2012; Malfasi et al. 2020).

(iii) Non-experimental introductions of non-native species persisting synanthropically

Our study identified 41 reports, concerning at least 22 species that have been or continue to be present synanthropically in research stations and other Antarctic facilities. However, the list of species known to have existed only synanthropically in Antarctica is probably not exhaustive, largely due to poor reporting, but does give an indication of the variety of species capable of persisting specifically in association with research stations and other Antarctic infrastructure. Hydroponic facilities and sewage treatment plants have been particularly prone to synanthropic infestation (Hughes et al. 2005; Bamsey et al. 2015; Bergstrom et al. 2018). Of particular current concern is *T. maculipennis* which, although originally largely assumed to have been associated with station sewage systems, has spread rapidly across several research stations and is now thought to survive and reproduce in the Antarctic environment (Volonterio et al. 2013; Remedios-De León et al. 2023; Poland 2024a).

Taxonomy of recorded non-native species

Established non-native species predominantly represent a small number of taxonomic groups (Fig. 2a). The deliberate introduction of species for experimental purposes involved plants almost exclusively, resulting in the large number of records of Magnoliopsida and Liliopsida and, to a lesser degree, Polytrichopsida (Smith 1996). In contrast, while unintentional introductions of Liliopsida and Magnoliopsida were recorded, the greater number have been of invertebrates, especially representatives of Collembola, Arachnida and Insecta. It is clear that, for most plant records, the plants no longer remain (Fig. 2b). This is because either (i) these were mostly experimental introductions involving small numbers of plants that were planted in small defined areas where eradication was simple to undertake and planned at the end of the experiment, or (ii) for non-experimental introductions, recorded plants were present as single specimens or in small numbers that were readily removed. The main exceptions here are the two more extensive *P. annua* populations at Admiralty Bay, King George Island, which persist despite on-going eradication efforts (Galera et al. 2017, 2019, 2021; Poland 2024b). In contrast, invertebrates once introduced tend to persist and there are no known attempts to eradicate them from the natural Antarctic environment.

Distribution over time

There has been a high degree of variability in the rate of reporting of non-native species occurrences over the past 80+ years since non-native species were first observed in Antarctica (Fig. 3a). The peak in the 1960s coincided with a series of transplantation experiments by researchers from the British Antarctic Survey at Signy Island, South Orkney Islands (Edwards and Greene 1973; Edwards 1980). The smaller peak from c. 2009 to 2017 coincided with increased survey effort that resulted from the profile given to non-native species issues following the International Polar Year 2007/08 project 'Aliens in Antarctica' (Chown et al. 2012) and, most notably, the survey by German researchers of soil microfauna at visitor locations around the western Antarctic Peninsula (Russell et al. 2013; it is appropriate to note that this is an institutional report and not a formally reviewed literature article). Indeed, most of the Arachnida and Collembola reported during the 2010 and 2011 'spike' were the result of the latter study and illustrate the information that can be generated if targeted research effort is funded (Fig. 3b). Identifying non-native invertebrates demands high levels of taxonomic expertise and the efforts made during the period 2009–2017 have not been repeated since. Since c. 2017, the number of new reports has been low, with most new records being of non-native insects living synanthropically within research station facilities.

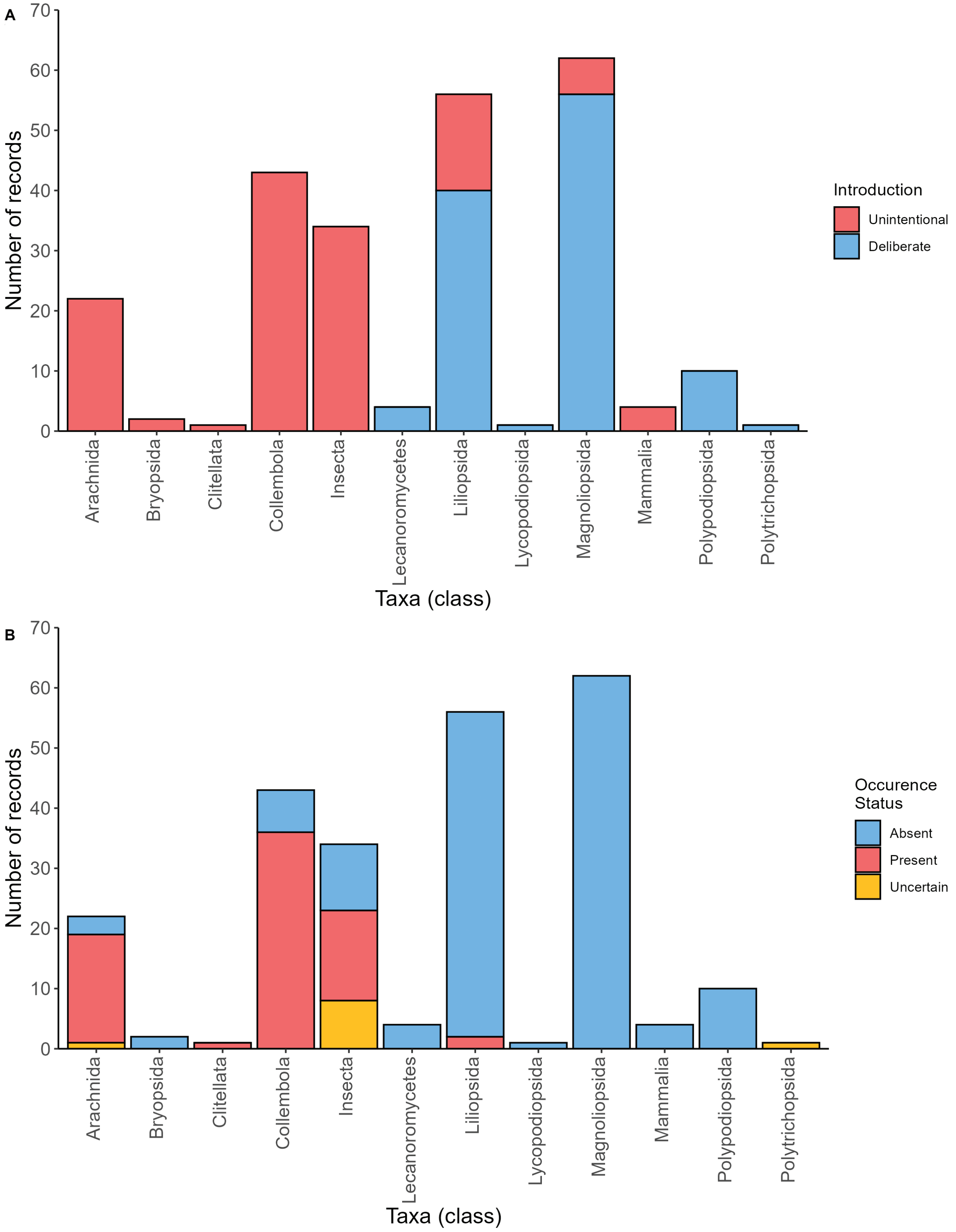

Figure 2. Number of records of different non-native species categorised to the taxonomic level of class that have established at different locations within the Antarctic Treaty area **A** data sub-divided based upon whether the introduction was deliberate or unintentional **B** data sub-divided based on the reported species' current occurrence status (absent/present/uncertain) in Antarctica.

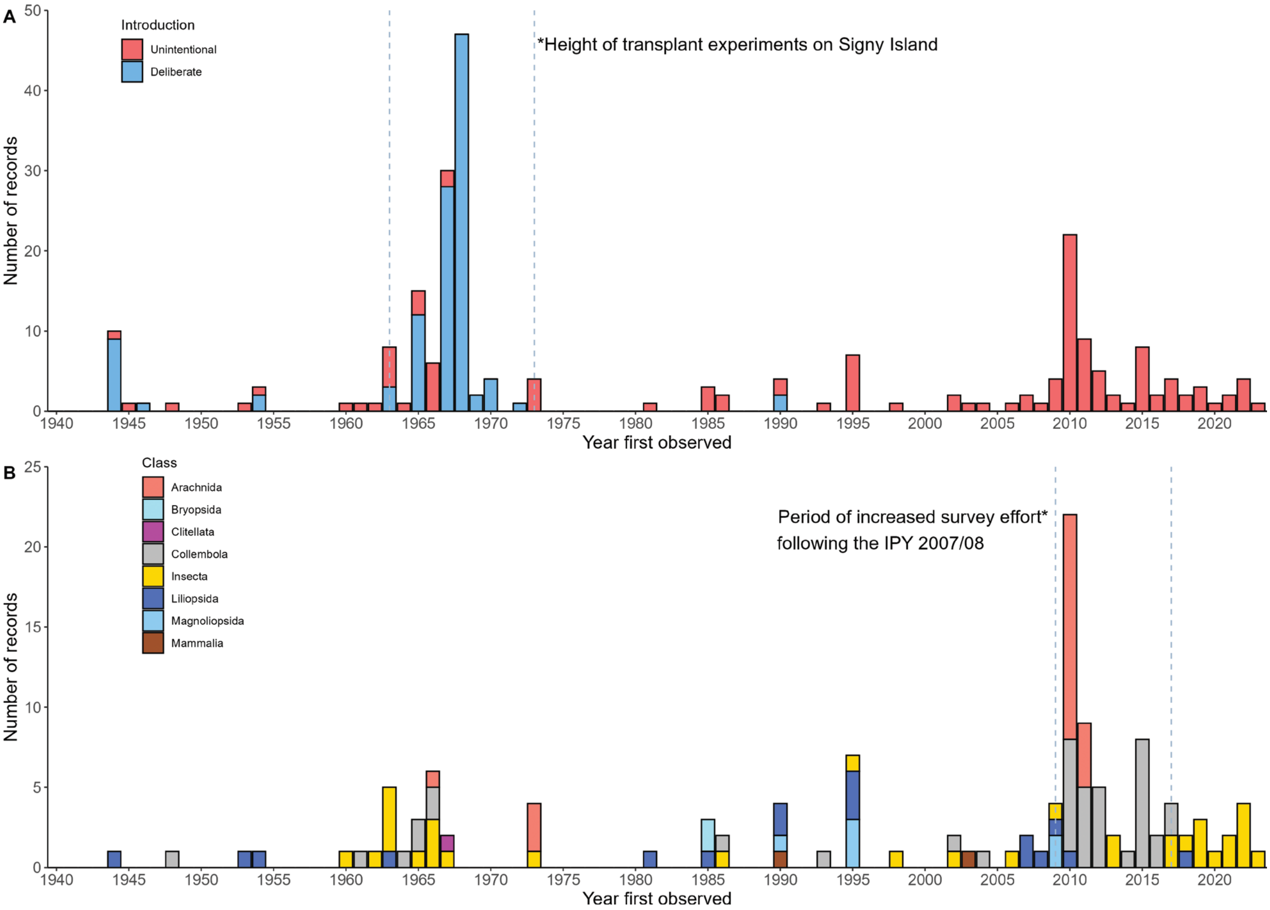

Figure 3. The year each reported non-native species occurrence was first observed in new locations within Antarctica (the three records from before 1940 are not displayed) **A** records classified by whether the introduction was deliberate or unintentional **B** records classified by taxonomic class of the species. Only records of species that were introduced unintentionally are included in B (therefore excluding the experimental introductions).

Association with human activities

The presence of Antarctic infrastructure increases the likelihood that non-native species will be found in the local vicinity. For example, c. 70% of reported non-native species existed either synanthropically within a research facility or had established within 1 km of infrastructure (Fig. 4). Of the species found in the natural environment, but within 1 km of research infrastructure, c. 75% were deliberate transplantation experiments that were subsequently removed (as were c. one third of reports found 5–10 km from infrastructure). Records of non-native species are also likely to be found near visitor landing sites commonly used by the tourism industry, with 85% of records (excluding synanthropic records) located within 1 km of a visitor site (recognising that a number of such sites are also close to research stations or foci of scientific research).

Fig. 5 shows the mean distance to the ten nearest biodiversity records for each non-experimental introduction and gives an impression of the survey effort at, and biodiversity knowledge of, each location. Smaller mean distances indicate a higher density of biodiversity records (and thus surveys and research undertaken at that location). The mean distance for biodiversity records in the biodiversity database (Terauds et al. 2025) to their ten nearest neighbours was 1.35 km. In comparison, over 90% of non-native species reports had ten biodiversity

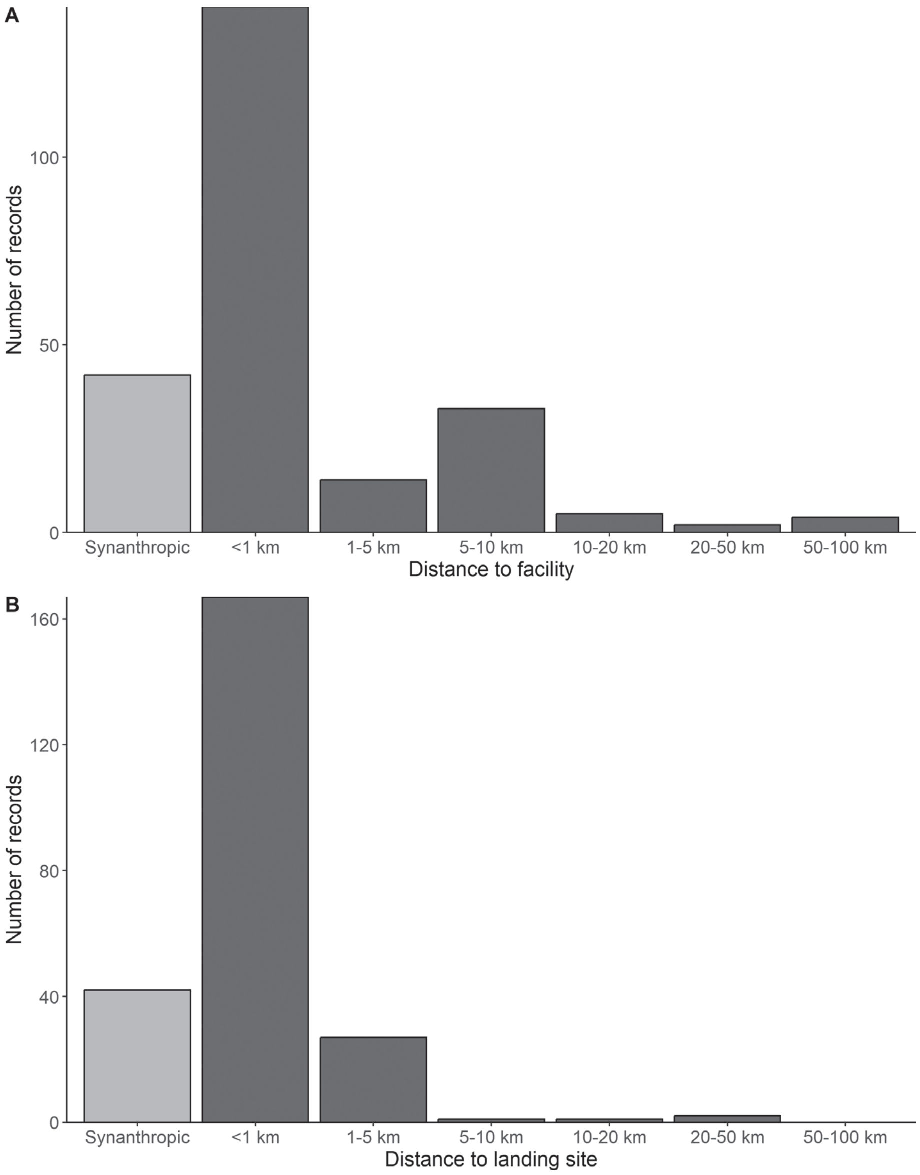

Figure 4. Distance of location of each non-native species record to **A** the nearest national Antarctic operator facility (e.g., research station, camp, airstrip, etc.), and **B** the nearest tourist landing site. Records classed as being located within Antarctic operator infrastructure (synanthropic records) are coloured light grey and labelled as 'Synanthropic'. Tourist landing sites can include national operator facilities, thus there are also synanthropic records included in (**B**). Records listed as < 1 km to facilities/landing site are in the vicinity of the infrastructure but are not inside it as such record would be classified as 'synanthropic'.

records within 700 m. These data indicate there has been a high level of survey effort at most locations where non-native species are detected, as could be anticipated given the close proximity of many records to research infrastructure (see Fig. 4). These data could reflect that non-native species are more likely to be detected in areas where there have been more biodiversity surveys and/or that they are more likely to be introduced at sites of high human activity. The higher density of biodiversity records near recorded non-native species could also indicate more suitable conditions for the establishment of native biodiversity more generally. However, it also highlights the dearth of both native and non-native biodiversity information from locations more distant from stations/visitor sites which, in the absence of data, make it impossible to know the extent of non-native species colonisation and distribution.

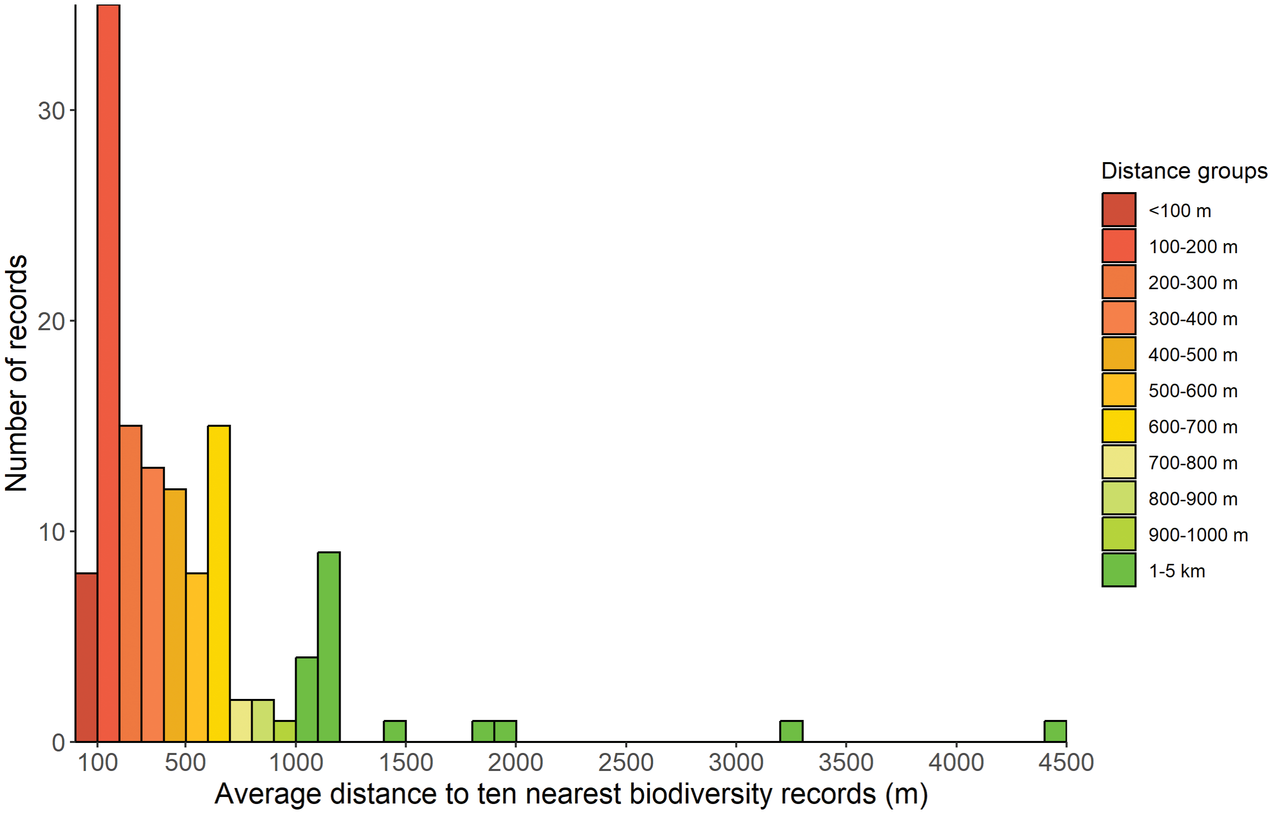

Figure 5. Average distance for each non-native species record location to the ten nearest biodiversity record locations. Only records classed as non-experimental introductions were included. The distance serves as a proxy of survey effort and biological knowledge of the site, where we assume that closer distances and higher densities mean that more research has been undertaken at the site.

Maximum survival time for currently present species

There are 20 species reported as currently present within the Antarctic Treaty area (Fig. 6), with some reported from more than one location (e.g., the springtail *Hypogastrura viatica* has been reported from 21 locations, more than any other Antarctic non-native species, albeit that many of these are from different parts of the same island, Deception Island). From Fig. 6 it can be seen that all established Antarctic terrestrial non-native species, except *P. annua*, are invertebrates, almost one third have been present in Antarctica for more than 50 years, and almost all have been present for more than 10 years. Only *P. annua* has been subject to any eradication efforts in the natural environment since it was first reported in the mid-1980s. More generally, apart from some species introduced during transplantation experiments, there are very few instances of established non-native species populations dying out without human intervention (although see Smith and Richardson (2011) and Hughes et al. (2017)).

Discussion

The introduction of non-native species, a proportion of which are likely to become invasive, presents one of the greatest threats to Antarctic terrestrial biodiversity today (Convey 2011; Convey and Peck 2019). Climate change, itself recognised as a global threat to biodiversity (but also, in isolation, likely to benefit in the short- to mid-term many native Antarctic terrestrial biota (Convey 2011; Lee et al. 2022b)) is also likely to exacerbate the threat of invasive species by increasing the likelihood

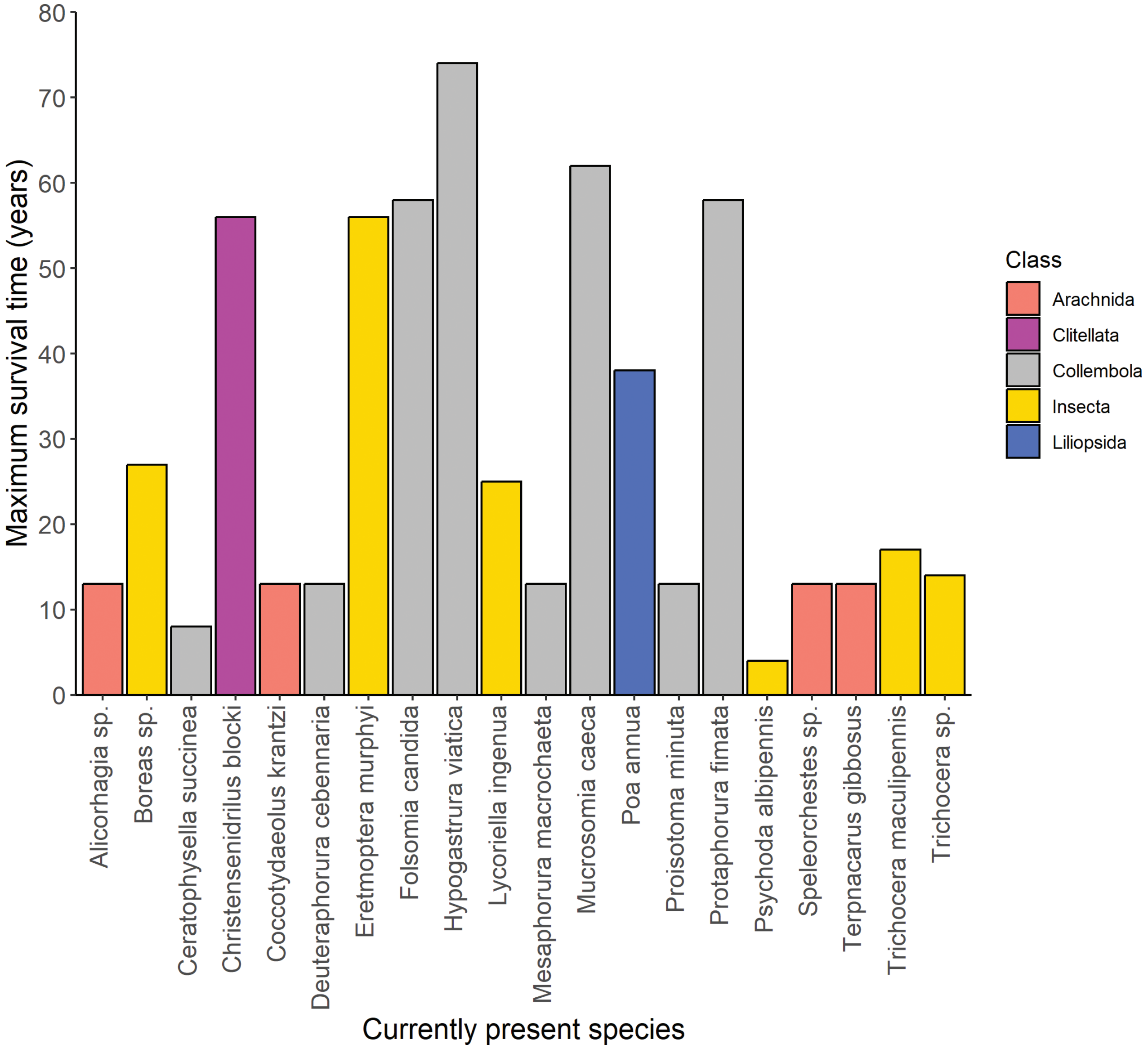

Figure 6. Maximum survival time of species currently known to be present within the Antarctic Treaty area.

of establishment (Chown et al. 2007; Beet et al. 2022; Siegert et al. 2023). The availability of datasets detailing non-native species introduction and establishment events is critical for policymakers to understand the scale of the risk and undertake appropriate policy responses (Hughes and Convey 2014; Hughes and Pertierra 2016; Antarctic Treaty Secretariat 2019; Remedios-De León et al. 2021).

Species introduced deliberately to the natural Antarctic environment during transplantation experiments

By far the largest group of records in our dataset related to the historical experimental introduction of plants from locations generally, but not always, beyond the Antarctic Treaty area in order to assess their survival under Antarctic conditions (Fig. 3a; for an overview, see Smith 1996). The longest experiments involving the greatest number of species were conducted by researchers from the British Antarctic Survey in ground adjacent to Signy Research Station, South Orkney Islands, during the 1960s and 1970s (e.g., Edwards and Greene 1973; Edwards 1980).

Some species transplanted from sub-Antarctic South Georgia, including *Acaena magellanica*, *A. tenera*, *Achillea millefolium*, *Phleum alpinum* and *Ranunculus repens*, survived for four years and some could likely have persisted for longer had they not been removed at the end of the experiment (Edwards 1980). Transplantation experiments have sometimes resulted in unintended consequences, with *P. pratensis*, at Cierva Point, and *E. murphyi* and *C. blocki*, at Signy Island, all being introduced in soil from beyond Antarctica and persisting long after the deliberately transplanted species were removed (Corte 1961; Burn 1982; Block and Christensen 1985; Dózsa-Farkas and Convey 1997; Pertierra et al. 2013, 2017b). Experimental transplantation of non-native species into the Antarctic natural environment has seldom if ever been undertaken in recent years (Fig. 3a), although transplantation experiments of native mosses over very short distances (a few tens of metres) have been done to assess the potential use of this method to minimize anthropogenic environmental damage in Antarctica (Câmara et al. 2021)

Non-experimental introductions of non-native species to the Antarctic natural environment

Our dataset shows that all non-native species thought to currently have reproducing populations in the natural environment in Antarctica are located in the western Antarctic Peninsula, South Shetland Islands and South Orkney Islands (generally known as the maritime Antarctic and incorporating the area covered by Antarctic Conservation Biogeographic Regions 1, 2 and 3; Terauds and Lee 2016; Fig. 1). Within this area, the most invaded location is Deception Island, where a combination of high human activity by the historical whaling industry, national operators and the tourism industry, plus the local presence of geothermally heated soils, generates an enhanced opportunity for species introduction and establishment (Greenslade et al. 2012; Hughes et al. 2015; Enriquez et al. 2019). Of the non-native species within Antarctica, *P. annua* on King George Island and *E. murphyi* on Signy Island might most reasonably be termed 'invasive' species, as defined by the CEP Non-native Species Manual ("non-native species that are extending their range in the colonised Antarctic region, displacing native species and causing significant harm to biological diversity or ecosystem functioning") (Antarctic Treaty Secretariat 2019). *Poa annua* has resisted on-going eradication efforts, probably because a seed bank is now present in the soil of Thomas Point near Arctowski Station (Galera et al. 2021; Poland 2024b). The persistence of this grass is of particular concern due to laboratory and field experimental studies that have concluded it can potentially outcompete the native higher plants (*Colobanthus quitensis* and *Deschampsia antarctica*), particularly under predicted climate change scenarios (Molina-Montenegro et al. 2019). Further research to understand the physiological limits of invasive species may allow future management attention to be focussed on particular regions and introduction pathways (see Duffy et al. 2017; Escribano-Álvarez et al. 2023). *Eretmoptera murphyi* is now accelerating in its spread from its original introduction site near Signy Research Station and may be responsible for a step change in increasing nutrient availability in local soils (Hughes and Worland 2010; Bartlett et al. 2020, 2023). Although the distribution of *E. murphyi* is currently restricted to a relatively small part of Signy Island, there are concerns that any further dispersal, by natural or anthropogenic means, could have substantial impacts upon terrestrial habitats and potentially the closely related endemic Antarctic fly *Belgica antarctica* across the western Antarctic Peninsula region (Hughes et al. 2013; Bartlett et al, 2021)

The only species occurrences in this group that have been eradicated or have had eradication attempts undertaken on them are plants. In some cases, considerable efforts have been taken to eradicate non-native plants (with mixed success) but, as far as we are aware, no effort has been made to eradicate or control non-native invertebrates beyond research facilities, even if they have often been known to be present at these sites for more than 10 years (Fig. 6). The lack of invertebrate eradication attempts in the natural environment is largely because the technology to do so has not been sufficiently developed and pesticide use for environmental management purposes is not listed as a legitimate use within the Environmental Protocol (Hughes and Pertierra 2016; Galera et al. 2017, 2021). Most efforts to eradicate *P. annua* within the Treaty area have involved the removal of single or small numbers of specimens from the vicinity of research stations or other sites by visiting researchers (Molina-Montenegro et al. 2012, 2014; Malfasi et al. 2020). In contrast, a vegetatively expanding patch of *P. pratensis* that was located on Cierva Point for almost six decades was eradicated by an international team of researchers over several days and involved the removal of over 500 kg of soil (Pertierra et al. 2013, 2017b). Annex II to the Environmental Protocol states that introduced non-native species shall be removed "unless the removal or disposal would result in a greater adverse environmental impact" (Article 4(5)); however, it may prove challenging to predict the potential impact of an introduced non-native species and adoption of a precautionary approach with the removal of the non-native species is likely to be the best approach (Hughes and Pertierra 2016).

Deciding the correct course of action when considering the removal of a newly discovered plant may not be straightforward. In early 2009, *G. nivalis* and *N. magellanica* were reported from Deception Island and the authors, KAH and PC, visited the location to assess the situation the following season (Smith and Richardson 2011). During the intervening period, the *G. nivalis* plants had been washed away by ephemeral streams; however, a single healthy specimen of *N. magellanica* remained, clearly several years old. Given that both species are native to Tierra del Fuego, it was not clear if the species' presence was a result of a natural colonisation event (in which case any plants should be protected), or an anthropogenic introduction event (in which case any plants should be eradicated) (see discussion in Hughes and Convey 2012). In the end, the remaining *N. magellanica* plant was removed as an example of application of the precautionary principle but, in the absence of any other evidence relating to the introduction or establishment event, it remains unclear whether or not the correct course of action was applied. Not least, a sole criterion of 'remove if close to an area of human activity' is inappropriate or simplistic, as it would likely apply to virtually all ice-free areas in the South Shetland Islands, the part of Antarctica closest to the nearest source of colonisers in southern South America and also the mildest part of Antarctica, hence the most likely to be successfully colonised by incoming natural propagules.

Non-experimental introductions of non-native species persisting synanthropically

Forty-one reports concerned at least 22 species that have been or continue to be present synanthropically in research stations and other human facilities. Even though there are few explicit records, we acknowledge that rats and mice have probably been introduced to Antarctica on multiple occasions by historical shipping and industrial operations, as well as early in the phase of research station development (Headland 2012) but have not survived. Today, enhanced hygiene practices and waste management means that opportunities for rodent establishment, if introduced, are likely to be much reduced. Many of the invertebrate species recorded have been associated with greenhouses and hydroponic facilities on Antarctic research stations (e.g., Bergstrom et al. 2018). Numerous reports of spiders, dipterans, collembolans and psocids in such facilities exist, but formal identification to species level has been relatively uncommon (Greenslade 1987; Smith 1996; AAD 1998; Bamsey et al. 2015). Such synanthropic establishments may be expected given the high rates and diversities of invertebrate introductions recorded at some stations located across Antarctica (Chwedorzewska et al. 2013; Houghton et al. 2016; Newman et al. 2018). Sewage treatment plants have also been subject to increasing reports of non-native species introductions with *Lycoriella ingenua* at Casey Station and *T. maculipennis* (and most recently *Psychoda albipennis*) recorded at an increasing number of treatment plants on King George Island, South Shetland Islands (Hughes et al. 2005; Volonterio et al. 2013; COMNAP 2019; Korea and Chile 2022; Hernandez-Martelo et al. 2024). Emerging reports that *T. maculipennis* is reproducing in the natural environment and the potential human-mediated or natural movement of this winged species to other Antarctic locations are major causes for concern and there now appears to be similar potential for *P. albipennis* (Potocka and Krzeminska 2018; Hughes et al. 2019; Remedios-De León et al. 2021; Hernandez-Martelo et al. 2024; Kang et al. 2024; Poland 2024a).

Status developments in the past decade

Since the publication of Hughes et al. (2015) details of 39 new records of non-native species observed across the continent have been published. All but two of these are from the South Shetland Islands and 21 are new Collembola records from targeted surveys by Enríquez et al. (2018; 2019) at different sites on Deception and Barrientos Islands. One of the new records is for *P. annua*, where a clump of two individuals was discovered on the Gourlay Peninsula on Signy Island (Malfasi et al. 2020). The clump was removed several days later in accordance with Annex II to the Protocol on Environmental Protection to the Antarctic Treaty. The clump was located more than 2 km from Signy Island research station (the closest facility, and also with no evidence of the species being present), suggesting the species may have reached this location via non-assisted dispersal in the region (Malfasi et al. 2020). Given the potentially drastic impacts *P. annua* could have on the environment, rapid detection and removal is essential, although such opportunistic observations also highlight the general lack of detailed expert survey effort across the entire region and, hence, lack of explicit knowledge of unsurveyed areas. One new record is for the synanthropic Collembolan, *Xenylla* sp., which was subsequently eradicated from hydroponic facilities on Davis Station, East Antarctica (Bergstrom et al. 2018). Thirteen records are for confirmed synanthropic insects (all Lepidoptera or Diptera). Of these, three are for the Indian meal moth (*Plodia interpunctella*) and one for the Mediterranean flour moth (*Ephestia kuehniella*), detected in three different research stations on King George Island and one further south at Yelcho Station on Doumer Island (Câmara et al. 2022; Benitez et al. 2024). All individuals seen were eradicated (or presumed eradicated), although this has not been confirmed. Two other records concerned the moth *P. albipennis*, whose increasing distribution on King George Island would benefit from further research to understand if the species can reproduce in the natural environment (Korea and Chile 2022; Hernandez-Martelo et al. 2024). While some earlier records of *T. maculipennis* are non-synanthropic but with no evidence of reproduction in the natural environment (e.g., Volonterio et al. 2013), six recent synanthropic records are for the fly having colonised station sewage treatment plants. One additional new record for *T. maculipennis* was within a refuge hut in ASPA 132 (before it was promptly eradicated), more than 9 km from its closest currently established population at King Sejong station (Korea et al. 2016; Remedios-De León et al. 2021; Argentina and Uruguay 2022). A recent non-peer-reviewed paper submitted to the CEP recorded *T. maculipennis* at several locations within ASPA 128 Western Shores of Admiralty Bay (Poland 2024a). At one location within the ASPA, Llano Point, the presence of larvae and pupae in the vicinity of penguin colonies indicated that the fly can survive and reproduce beyond station confines. If accurate, this is probably the largest and most concerning non-native species development within the past decade (Remedios-De León et al. 2021). The continued expansion of *P. annua* at Thomas Point into areas of native plant communities, despite substantial eradication efforts, is the other major concern (Poland 2024b).

Developments in non-native species policy and response

Up until the end of the first decade of the 21st century, targeted survey effort to identify non-native species was lacking, with most introduced populations identified by chance or during other survey work (Fig. 3). The issue of non-native species introductions and biosecurity received an increased profile within the ATCM following the publication of results of the International Polar Year 2007/08 'Aliens in Antarctica' research project (SCAR 2010; Chown et al. 2012; Huiskes et al. 2014). Subsequently, more targeted surveys were undertaken, particularly for non-native invertebrates in the vicinity of frequently visited sites, and new non-native populations were found (e.g., Russell et al. 2013; Enríquez et al. 2018, 2019). However, with the spread of large (relative to other native species), persistent and easily dispersed non-native species, such as *T. maculipennis* and *P. annua*, in the past decade or so, the issue of non-native species management and control/eradication has increased in profile (Hughes and Pertierra 2016; Remedios-De León et al. 2021). Some Parties have allocated resources to undertake research and initiate management and control of these species with varying degrees of success (Galera et al. 2017, 2019, 2021; Potocka and Krzeminska 2018; Kang et al. 2024; Korea 2024). However, in recent years, fewer specific surveys targeting new non-native species have been reported in the academic literature, and most reported introductions have been in the immediate vicinity of research stations or of species living within research facilities (Korea and Chile 2022; Benitez et al. 2024). It is possible that some Parties may be using their available budgets to manage existing non-native species with monitoring for new species consequently falling down the priority list. Nevertheless, the association of established non-native species with national operator infrastructure and tourist visitor sites (Fig. 4) highlights the need for on-going and enhanced biosecurity precautions that are applicable to all human activities in the region. The CEP Non-native Species Manual identifies three major components of a non-native species management framework: Prevention, Monitoring and Response, and all are essential if Antarctic environments are to be adequately protected. Assuming that governments allocate resources to their national Antarctic programmes to address non-native species issues, it may be a challenge to determine how best to divide this funding to deliver (i) effective biosecurity practices along the supply chain (Prevention), (ii) monitoring for new non-native species in Antarctica (Monitoring), and (iii) control and/or eradication of established non-native species and delivery of research to identify practical methods to respond to these introductions (Response). Nevertheless, in an Antarctic context, the CEP non-native species manual acknowledges that resources targeted towards prevention of species introduction and associated biosecurity measures deliver the greatest conservation benefit compared with other management responses (Antarctic Treaty Secretariat 2019).

It is notable that non-native plants have generally been eradicated, but the difficulty in delivering the full eradication of *P. annua* at Admiralty Bay is a major cause for concern, and it can only be hoped that Poland maintains its on-going efforts to control the grass (Galera et al. 2017, 2019, 2021; Poland 2024b). Also of concern is the almost universal failure of Parties to control or eradicate any non-native invertebrates that have established in the natural environment, with some of these species now having persisted in Antarctica for several decades. Investment in research to identify practical methods to respond to these introductions is urgently needed, although many such invertebrates may now be beyond any practical form of control, as has also been recognised for a number of non-native species on sub-Antarctic islands (e.g. South Georgia (Black 2022)).

Final remarks

The records presented here provide evidence regarding the number, diversity and spatial distribution of species introductions leading to short or long-term establishment in the Antarctic Treaty area. For most of the 19th and 20th centuries, precautions taken to prevent the introduction of non-native species were few or non-existent. Today, the Antarctic tourism industry, under the guidance of IAATO, generally employs extremely high standards of biosecurity, commensurate with their concerns for preserving the Antarctic environment and the image of organisational environmental awareness and responsibility that they wish to promote to their clients (IAATO 2023, 2024). National Antarctic programmes, in general, manage much more complex logistical operations in Antarctica than does the tourism industry and may struggle to achieve equivalent high standards across their range of operations. Further, it is also likely that levels of awareness of non-native species issues differ across national programmes, alongside the level of implementation of biosecurity measures, despite the best efforts of COMNAP and SCAR (COMNAP 2015; COMNAP and SCAR 2019). The CEP is responsible for providing advice to the ATCM on issues relating to non-natives species. However, in recent years, despite numerous papers by Antarctic Treaty Parties describing the challenges of addressing non-native species within the Treaty area (e.g., Poland 2024a, b), there have been few initiatives emanating from the CEP to further enhance biosecurity, or to understand how effectively national operators are implementing biosecurity measures, despite this being given high priority on the CEP Five-Year Work Programme (Antarctic Treaty

Secretariat 2024b). It is hoped that the information made available in this dataset (https://doi.org/10.5285/afeb9f5e-bd69-4e3d-9d50-e935134f4c78) and associated online application (https://saer-non-nativespecies.data.bas.ac.uk/) will demonstrate clearly the extent and increasing seriousness of the challenges created by non-native species in Antarctica and that accelerated policy development and management action will result (Lee et al. 2022b).

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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

KH: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original draft, Writing - Review and Editing. PC: Conceptualization, Methodology, Validation, Investigation, Writing - Original draft, Writing - Review and Editing. JL: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original draft, Writing - Review and Editing, Visualization.

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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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Research Article

Warming and latitude shape the non-consumptive effects of native and invasive alien crayfish predators on damselfly prey

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Abstract

There is increasing concern that the effects of biological invasions may be magnified by other human-induced global changes. Here, we compare the non-consumptive effects imposed by invasive vs. native predators and how these (differential) responses to both predator types depend on warming and prey latitude. We raised damselfly larvae from central- and high-latitudes in incubators under two temperatures (current [20 °C] and warming [24 °C]) and further exposed them to one of three predator cues: noble (native), signal (invasive at both latitudes) and spiny-cheek (invasive at central- but absent at high latitudes) crayfish. Growth rate increased in central-latitude but decreased in high-latitude prey in response to both noble and signal crayfish. The spiny-cheek crayfish only reduced growth rate in high-latitude prey. Cues from all three crayfish species generally caused a higher net energy budget, but only under warming. Our results demonstrated that high-latitude prey were able to recognize a novel invasive predator (spiny-cheek crayfish) cue, and revealed differential growth responses of central- and high-latitude prey toward the shared invasive predator (signal crayfish). Our data provide rare support for the concern that global change factors may magnify the impact of both native and novel invasive predators.

Key words: Energy allocation, global warming, invasive predators, *Ischnura elegans*, latitudinal gradient, non-consumptive effects

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Introduction

Biological invasions are a key component of human-induced biotic global changes and pose a worldwide threat to biodiversity and ecosystem functioning (Bellard et al. 2016). Specifically, the introduction of novel (invasive or not) predators may have a profound impact on native prey because 1) they may lack the ability to recognize and respond to these predators, 2) may respond inappropriately or 3) may respond appropriately but are outsmarted by predators (naïve prey hypothesis [NPH], Cox and Lima 2006; Banks and Dickman 2007). The NPH was tested in various ecological contexts especially when exotic predators show little or no similarities with native predators, e.g. in term of relatedness, appearance or predation skills (Carthey and Banks 2014; Anton et al. 2020). The NPH was supported by several empirical studies, for example in mussels facing an invasive crab (Freeman and Byers 2006), native fish prey facing an invasive lionfish (Anton et al. 2016) and insects facing an invasive fish (Townsend 2003). Prey naïveté can ultimately lead to declines and even extinctions of populations of native prey if these fail to recognize invasive predators (Carthey and Banks 2014). Hence, according to the NPH, invasive and native predators showing different degrees of similarity are likely to trigger differential responses in native prey. This differential effect is likely to become smaller when the evolutionary history of co-occurrence with an invasive predator species increases (Anton et al. 2020).

While the studies on novel invasive predators (invasive predators, hereafter) on prey mainly focused on direct consumptive effects, predators may also affect prey through indirect non-consumptive effects by modifications in prey behaviour, physiology and life-history traits (Hawlena and Schmitz 2010). Indirect effects are important to study as these may contribute as much or even more to prey population dynamics than direct effects (Preisser et al. 2005). In extreme cases, indirect effects can be absent in response to an invasive predator, as has been documented, for example, in shell thickening in molluscs (Freeman and Byers 2006) and refuge use in crustaceans (Martin 2014). Other studies did demonstrate indirect effects of invasive predators but these differed from the indirect effects imposed by native predators with, for example, an increased oxidative stress in amphibians (Pinya et al. 2016) and slower development in damselflies (Antoł and Sniegula 2021) when exposed to an invasive compared to a native predator. A largely ignored type of indirect effects are bio-energetic responses to predation risk. Understanding how predation risk shapes the available energy reserves of prey and their consumption, and especially their balance is important as this net energy budget may have fitness consequences and can explain life history responses to stressors in prey (e.g. Verheyen and Stoks 2020); as such it may improve mechanistic insights in the impact of native vs. invasive predators on local prey populations.

There is increasing concern that the effects of invasions may be reinforced by human-induced abiotic factors such as those related to climate change (Lopez et al. 2022). Knowing when and how the effects of invasive predators on prey are modified by such global change factors are barely understood, yet crucially needed for conservation practices (Anton et al. 2020; Lopez et al. 2022). One particular abiotic factor that may modulate prey responses to predation risk, including the effect of invasive predators, is temperature. The general idea is that under warming, prey may take greater risks to meet their increased energetic demands leading to higher encounter rates with predators (Lienart et al. 2014; Mitchell and Harborne 2020). For example, growth acceleration and increased metabolic rates were observed when predation risk imposed by native predators was combined with warming in damselflies (Stoks et al. 2012; Janssens et al. 2015). But the opposite pattern was also found with, for example, lower growth when predation risk was combined with warming in the damselfly *Enallagma vesperum* (Culler et al. 2014). Native and invasive predators may potentially trigger differential indirect effects under warming but the direction and the strength of the response remains unclear, asking for empirical studies. One obvious reason for a differential response to warming between both predator types is when prey never reacts to invasive predators (irrespective of temperature) while the response to native predators increases with warming. More general, whenever indirect effects of invasive predators differ from those of native predators (as predicted by the NPH, Cox and Lima 2006; Sih et al. 2010), these differences can be expected to be magnified by warming, especially when responses to temperature are non-linear.

One rare study that integrated both types of predators and warming, demonstrated stronger effects of an invasive compared to a native predator in term of delayed egg developmental time under warming compared with ambient conditions in the damselfly *Ischnura elegans* (Amer et al. 2024).

Another factor that may shape indirect prey responses to predators in general is the latitude of origin of the prey. This is because prey species often show different life history strategies linked to thermal adaptation along latitudinal gradients that may affect their response to predators and its dependence on temperature (Freeman and Byers 2006; Debecker and Stoks 2019; Palomar et al. 2023; Wos et al. 2023). For instance, in temperate regions many insects complete more generations at lower latitudes, typically resulting in stronger seasonal time constraints and faster life histories (Zeuss et al. 2017). Time-stressed individuals should invest more energy into growth and development to reach a specific mass and size before a critical time point (at least in overwintering cohorts) (Ludwig and Rowe 1990). Consequently, prey responses to predators are expected and shown to be weaker under time constraints, as shown in insects (Stoks et al. 2006) and amphibians (Altwegg 2002). Given the overall different prey responses to native predators at different latitudes, the same reasons as given for warming may potentially cause the impact of invasive predators to differ between prey of different latitudes, especially when the evolutionary history with an invasive predator differs between prey from different latitudes.

Here, we tested the NPH in the context of global warming and included an intraspecific component to assess latitudinal differences in prey responses. Specifically, we evaluated the indirect effects imposed by native vs invasive predatory crayfish on the growth and bio-energetic traits of the native damselfly, *I. elegans*, during its larval stage, and how these patterns were shaped by warming and prey latitude of origin. We used three crayfish species differing in their invasive status at the two studied damselfly latitudes of origin: the noble crayfish (*Astacus astacus*) which is native at both latitudes, the invasive signal crayfish (*Pacifastacus leniusculus*) which invaded both latitudes since the 1970s (Kouba et al. 2014), and the invasive spiny-cheek crayfish (*Faxonius limosus*) which is non-native at both latitudes but only invaded the central latitude where it was introduced at the end of the 19th century. We reared larval damselflies from replicated ponds at high and central latitudes in the laboratory and exposed them to native or invasive predator cues at current (20 °C) and warming (24 °C) temperatures. We measured larval growth rate, and a set of fitness-related bio-energetic parameters that capture energy availability and consumption, and are known to be affected by predator cues in damselfly larvae (Janssens et al. 2015).

Based on the theoretical predictions that native prey would recognize invasive alien predators which whom they share an evolutionary history (Anton et al. 2020) and previous case studies (Palomar et al. 2023; Wos et al. 2023; Amer et al. 2024), we expected *I. elegans* to recognize the three predator species, yet with a different response between native and invasive predators. In general, we expected damselfly prey to react to predators with an accelerated growth rate (Stoks et al. 2012), a faster metabolism and lower energy storage (Stoks et al. 2005a, Van Dievel et al. 2016). According to the NPH, as the damselfly species shared a longer evolutionary history with their native predator, we expected a stronger response for growth and bio-energetic variables in response to the native than the invasive predators. As the degree of relatedness between native and invasive predators decreases, we may expect smaller differences in the responses between both predator types. We also expected the invasive predator effects on prey to be more pronounced at 24 °C compared to 20 °C, and the effects induced by the invasive signal crayfish (present at both latitudes) to be more pronounced in the high-latitude prey populations (compared to the central populations) as these are less time-stressed. Yet, for the invasive spiny-cheek crayfish (only present at the central latitude) we expected stronger predator effects at the central latitude because of the latitude-specific evolutionary history with this invasive predator.

Methods

Part of the phenotypic data (growth rate data) used in the present study was used in another article focusing on the effects of urbanization and predator cues (spiny-cheek crayfish only) on *I. elegans* collected in urban and rural ponds at different latitudes (Palomar et al. 2023). The current study addresses novel questions by focusing on the indirect effects induced by native vs. invasive predators and includes novel datasets related to noble and signal crayfish, and to physiological traits.

Description and sampling of Ischnura elegans

The study species, *I. elegans*, is a common damselfly species in Europe (Dijkstra and Schröter 2020). At central latitudes, such as Poland, populations are typically uni- and bivoltine, i.e., have one or two generations per year, respectively. At higher latitudes, including Sweden, populations generally display a uni- and semivoltine pattern, i.e. one or two years are required to complete a single generation (Corbet et al. 2006; Norling 2021). Mating pairs of *I. elegans* were captured using insect sweep nets, with adult females obtained from two ponds in southern Sweden (hereafter, high latitude) and two ponds in southern Poland (hereafter, central latitude) (Suppl. material 1: table S1) on June 22–23, 2021, following the protocol outlined in (Sniegula et al. 2020). The distances between the two central- and two high-latitude ponds were, respectively, 73 km and 18 km. In total, 10 adult females were captured per pond (10 females × 2 ponds × 2 latitudes = 40 females). Adult females were individually housed in plastic cups with perforated lids and wet filter paper at ~22 °C under natural daylight (photoperiod). Each female laid one egg clutch giving a total of 40 clutches.

Description and sampling of crayfish species

We used chemical cues from three crayfish species: one native and two invasive species. The noble crayfish (*A. astacus*) is native and has a wide distribution in Europe. Until 2015, the crayfish was present in one of the central latitude ponds (Krakow pond, Suppl. material 1: table S1, Stanek et al. 2015). The approximate distance between sites where the noble was reported during the study season and ponds where central and high latitude damselflies were collected is ca. 40 km (Maciej Bonk and Rafał Maciaszek, personal comm.) and 9 km (Artportalen 2024). The noble crayfish were collected in a private pond in western Poland (51.653667°N, 16.981194°E) and in Gróbka River (49.964889°N, 20.501500°E).

The signal crayfish (*P. leniusculus*) native to North America, was introduced to Europe during the 1960s and is largely present in Scandinavia and in Poland (Kouba et al. 2014). The approximate distance between the closest crayfish population and the ponds where central and high latitude damselflies were collected is ca. 110 km (Maciej Bonk and Rafał Maciaszek, personal comm.) and ca. 3 km. The signal crayfish were collected in Obłęski Lake in northern Poland (54.242889°N, 16.917278°E).

The spiny-cheek crayfish (*F. limosus*) originates from North America. Introduced to central Europe at the end of the 19th century, it has become the predominant crayfish species in EU countries, including Poland, with the exception of Scandinavian countries where the species is absent (Kouba et al. 2014; Artportalen 2024). The spiny-cheek crayfish is present in the Vistula river located at 400 m from the sampled pond in Krakow (Orłowska and Romanowski 2023). The spiny-cheek crayfish were collected in Kryspinów Lake in southern Poland (50.050128°N, 19.789125°E).

Because of its dispersal ability, *I. elegans* may have encountered some crayfish predators, though exposure varied by region. The species can disperse across multiple sites within a region (Conrad et al. 1999; Gall et al. 2017), which was supported by genomic studies demonstrating high gene flow at a local scale (Babik et al. 2023). While central-latitude populations are near spiny-cheek crayfish, they are ca. 40 and 110 km from reported noble and signal crayfish populations, making encounters more likely for noble than signal. In contrast, high-latitude populations are close to noble and signal crayfish (ca. 9 and 3 km), but far from spiny-cheek crayfish (ca. across the Baltic See and 210 km from Denmark; Ion et al. 2024).

The NPH states that a closer evolutionary distance between native and invasive predator may help their recognition by native prey (Anton et al. 2020). Previous genetic and phylogenetic analyses established the relationship between these three crayfish species. The noble and signal crayfish belong to the same family (Astacidae) and are phylogenetically closer to each other than to the spiny-cheek crayfish (Cambaridae) (Owen et al. 2015). The two families Astacidae and Cambaridae form a paraphyletic group (Owen et al. 2015).

A couple of weeks before the experiment, crayfish were collected in the field (May 2021) and acclimated in laboratory conditions. The crayfishes were housed in aquaria (l = 69 cm; w = 34 cm; h = 39 cm) filled with 50 L of dechlorinated tap water, at a constant temperature of approx. 20 °C. We placed three crayfishes of similar size and mass per aquaria. They were fed with fish food pellets twice per week and live worms once per week. All crayfish species were collected with permission from the pond owner and the Regional Directorate for Environmental Protection in Kraków (ref. DZP-WG.6401.147.2021. TŁ for noble crayfish; ref. OP-I.672.8.2020.MK1 for signal crayfish; ref. OP.672.4.2021.GZ for spiny-cheek crayfish).

Experimental procedure

Upon arrival at the laboratory, egg clutches were placed in an incubator with a temperature of 22 °C and under a photoperiod of L:D 20:4 h. After 15–17 days, eggs hatched and the experimental part started and was divided into three phases: two pre-treatment phases 1) The pre-winter phase during which all larvae underwent uniform pre-winter conditions, and 2) the winter phase during which all larvae underwent uniform winter conditions, and finally 3) the treatment phase during which larvae were exposed to the temperature and predator treatments (Fig. 1).

1) Pre-winter phase

Eight containers (22 × 16 cm, height 11 cm) and filled with 1500 mL of dechlorinated tap water were prepared for each pond matching the number of treatments (two temperatures crossed with four predator cue treatments [noble, signal, spiny-cheek crayfish and control]). The containers were placed in an incubator at 22 °C and 20:4 h L:D. These conditions induce high development and growth rates in larvae from both latitudes, particularly during post-winter rearing (Norling 2021). Under 20:4 h L:D, central latitude individuals may be more time-stressed compared with high-latitude individuals, however, our results did not reveal higher mortality at central latitudes and confirmed the expected difference in growth rate between latitudes (see results part). For each pond, once the majority of the ten clutches hatched, we randomly selected 4 larvae from each clutch that were placed into each of the eight containers totalling 40 larvae per container (4 ponds × 10 clutches × 4 larvae per clutch × 8 treatments = 1280 larvae at the beginning of the experiment). A plastic structure was provided in every container to minimize cannibalism among the larvae. Larvae were fed ad libitum with laboratory-cultured *Artemia* nauplii, twice a day on weekdays and once a day on weekend days. After three weeks, live *Daphnia* sp. were added to the feeding regime twice a week until autumn conditions were introduced, involving gradual change from summer to winter temperatures and photoperiods (described below). Container positions were randomized weekly within the incubator.

2) The winter phase

On 6 August 2021, approximately four weeks after larvae had hatched, simulated autumn temperature and photoperiod (thermo-photoperiod hereafter) were initiated. Three weeks later, winter conditions were simulated. This gradual reduction of the initial thermo-photoperiod 22 °C and 20:4 h L:D to 6 °C and 0:24 h L:D mimicked autumn and winter conditions in nature (Fig. 1). During the simulated winter, larvae were fed once a day, three times a week with *Artemia* nauplii.

3) Treatment phase

On 22 November 2021, immediately after the simulated winter, the treatment application phase started. All surviving larvae (total of 386 across all containers) were transferred to individual 200 mL cups, filled with 100 mL of dechlorinated water, and placed in an incubator at 10 °C and 4:20 h L:D. Over a two-day interval, the thermo-photoperiod was gradually increased to 20:4 h L:D and to the respective temperature treatment: 20 °C and 24 °C (Fig. 1). Water temperatures were selected based on dataloggers placed in three of our four ponds, estimation of water temperature for one pond (Lake model Flake; Mironov 2008) and on previous studies including our sampled ponds (Suppl. material 1: fig. S1) (Palomar et al. 2023; Wos et al. 2023). The temperature in high- and central latitude ponds fluctuated around 20 °C during the peak of larval development in late spring/early summer. Temperatures of 24 °C currently occur at both latitudes but infrequently. We established 20 °C as the current mean temperature, and 24 °C, corresponding to the predicted mean temperature by 2100 under the SSP8.5 scenario (Masson-Delmotte et al. 2021). Hence, the 24 °C treatment both reflects a warmer period in the study ponds at this moment, and the predicted future mean temperature in the study ponds.

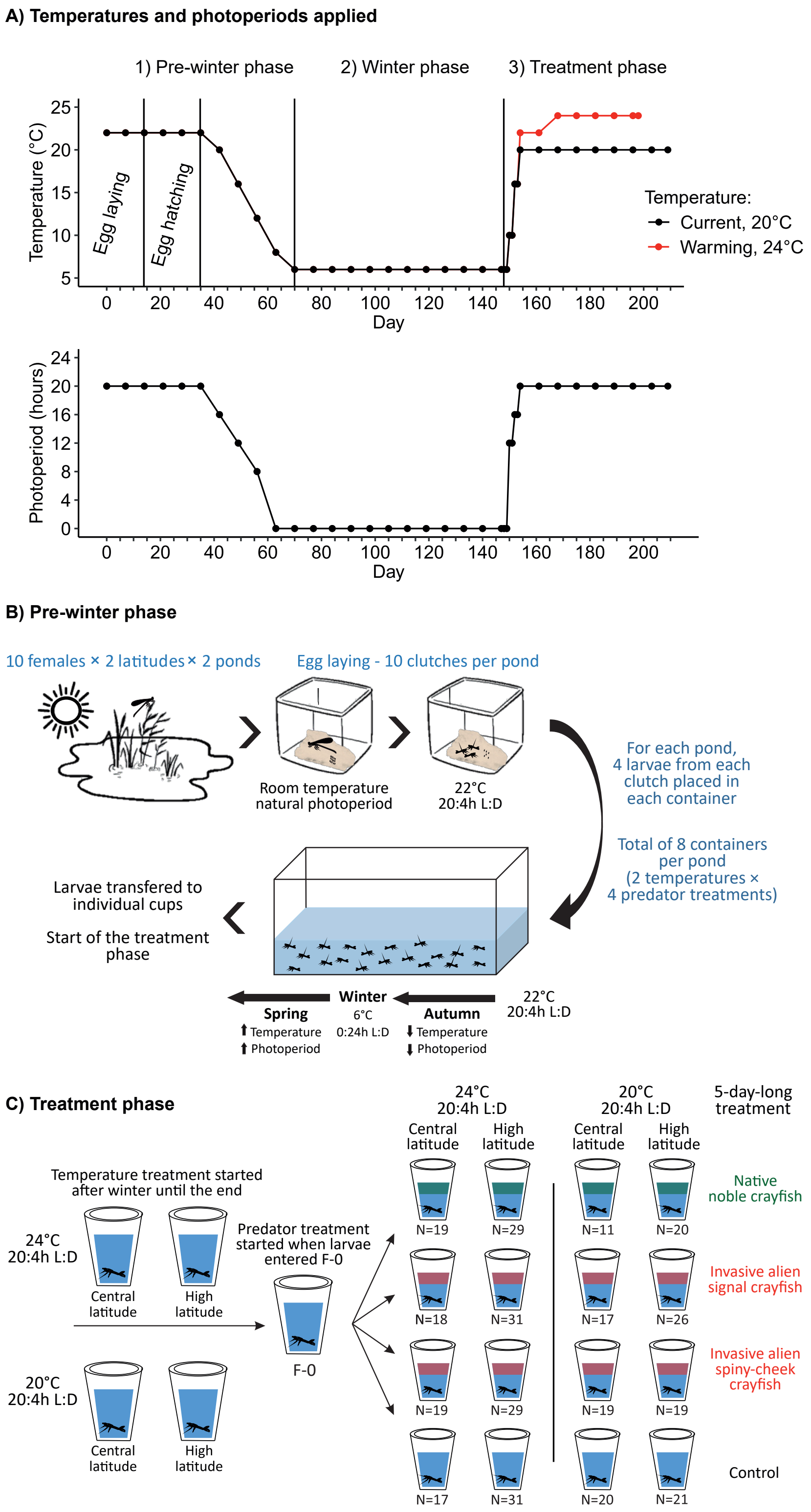

Figure 1. Summary of the experimental procedure **A** timeline of the experiment. The upper plot shows the variation in temperature over time including the different phases of the experiment. The lower plot shows the variation in photoperiod **B** for each pond, we collected 10 females (= clutch) and prepared eight plastic containers corresponding to the different treatments (2 temperatures × 4 predator treatments). For each pond, we placed four larvae from each family in each of the eight containers, totalling 40 larvae per container (4 larvae × 10 clutches). Larvae were kept in these containers during simulated pre-winter and winter periods. After winter, larvae were individualized in plastic cups and the experimental (temperature and predator cues) treatments started **C** details on the treatment application phase. Once larvae were split into individual cups, we started the temperature treatment. When larvae entered the final instar prior emergence (F-0), we crossed the temperature treatment with a five-day-long predator cue treatment (absence vs presence).

Throughout the treatment application phase, larvae were fed daily with *Artemia* nauplii. Given the different latitudes of origin and the influence of rearing temperature on larval development rate, larvae reached the F-0 instar at different dates and, consequently, were exposed to the post-winter temperature treatment for different durations (thermal exposure hereafter). When larvae entered the F-0 instar, the sex of each individual was identified.

Upon entering the F-0 instar, the thermal treatments were crossed with a five-day-long predator cue exposure treatment to one of the three crayfish species cues or to the control. Exposure of F-0 instars to a 5-day long treatment minimizes the chance of habituation and any effects on this instar are most likely to carry over to the adult stage, hence have fitness implications. Water samples collected from crayfish or control aquaria were warmed to the target temperature (20 °C or 24 °C). The water level in each cup was reduced to 67 mL and refilled with 33 mL of water from the crayfish aquarium (with predator cue) or the control aquarium (without predator cue). Cups were refilled every second day to maintain relatively constant predator cue levels, considering the biodegradation time of chemical predator cues (Van Buskirk et al. 2014). Previous experiments have shown that chemical predator cues affect damselfly life history traits, even during short exposure periods (13 days in Antoł and Sniegula 2021; 3–9 days in Van Dievel et al. 2016).

Response variables

Growth rate

In total, we quantified the growth rate of 346 larvae (Suppl. material 3). Larval growth (mass increase) has been considered a proxy for fitness in damselflies (De Block and Stoks 2008; Siepielski et al. 2020). When larvae entered F-0 and before the application of the predator cue treatment, we measured survival, and wet mass (mg; mass $_{F\text{-}0}$) with an electronic balance (Radwag AS.62). After the 5-day exposure to a predator cue, we measured the wet mass again ($\text{mass}_{\text{final}}$) and calculated the growth rate over the 5-day period as $[\ln(\text{mass}_{\text{final}}) - \ln(\text{mass}_{F\text{-}0})]/5$, as in (McPeek et al. 2001).

Physiological parameters

We quantified a set of bio-energetic parameters on 315 of the 346 larvae measured for growth rate: the activity of the electron transport system (ETS) and the concentrations of the three major energy storage molecules: the fat, sugar and protein contents. These physiological parameters were subsequently used to obtain the cellular energy allocation (CEA), an estimate of the net energy budget calculated as the sum of energy available (Ea; energy stored in proteins, sugars and lipids) divided by the energy consumed (Ec; estimated based on the ETS activity). Detailed descriptions of the physiological analyses are available in Suppl. material 1: file S1.

Statistical analyses

All statistical analyses were performed using R (R Core Team 2013, RStudio Team 2015 version 2024.12.0). For survival, we ran a model with latitude, temperature and their interaction as predictors; population nested in latitude was added as a random factor. Survival was considered as a binomial variable. We used generalized linear mixed-effects models (GLMMs; Magnusson et al. 2017). P-values were obtained using the Wald chi-square test (Wald X^2) implemented in the car package (Fox and Weisberg 2019). For the analysis of growth rate, we first ran a general model including the following predictors: sex (male vs. female), predator (control vs. noble vs. signal vs. spiny-cheek), temperature (20 °C vs. 24 °C), latitude (central vs. high latitude), and all possible interactions. As larvae were exposed to the post-winter temperature treatment for different durations, we used thermal exposure as a covariate. Population nested in latitude was added as a random factor. Significance of random effects was tested using likelihood ratio test by comparing the full (with random factors) versus reduced (without random factor) models using 'lrtest' (lmtest package version 0.9–40; Kuznetsova et al. 2017). We computed the proportion of variance for each significant random factor using the intraclass correlation coefficient, we reported the unadjusted coefficient to take into account the variance of fixed effects (performance package; Lüdecke et al. 2021).

As the variable predator contains four levels, it may be difficult to detect significant patterns especially if growth rate would be differentially affected by the three predator species. Hence, we also ran individual models for each predator species separately where we specifically compared the predator cues of a given crayfish species vs. the control. For this, we performed a model selection analysis (MuMIn package; Bartoń 2024) to select the models for each predator vs control comparison. We included in the initial model the following variables: population (two ponds per latitude) and all possible interactions between sex, predator cue treatment (one crayfish species vs control), temperature and latitude; thermal exposure was added as a covariate. Selection of the average model was based on the corrected Akaike's information criteria for small sample size (AICc) using Delta < 2 (Suppl. material 1: table S3) (model.avg function in MuMIn package; Bartoń 2024). Then, we ran the selected average model using generalized linear mixed-effects models (GLMMs; Magnusson et al. 2017).

For the analyses of the three focal physiological parameters, we first ran a model for Ea, Ec and the integrated parameter CEA including the following predictors: sex (male vs. female), predator (control vs. predator cue), temperature (20 °C vs. 24 °C), latitude (central vs. high latitude), and all possible interactions; thermal exposure was added as a covariate and population nested in latitude was added as a random factor. The four individual "raw" physiological parameters: ETS activity and the three variables related to energy storage (fat, sugar and protein contents) were analysed using multivariate statistics. First, we ran a Multivariate Analysis of Variance (MANOVA) for each predator treatment vs. control to identify and select the relevant predictors affecting the physiological parameters. The MANOVA model included the four physiological parameters (log-transformed) and the following predictors: sex (male vs. female), predator (control vs. predator cue), temperature (20 °C vs 24 °C), latitude (central vs. high latitude), and all possible interactions between predator, temperature and latitude; thermal exposure was added as a covariate. The significant predictors revealed by MANOVA were subsequently used in a Constrained Correspondence Analysis (CCA; vegan package; Oksanen et al. 2013) to evaluate and to visualize their contribution to the physiological variation. In addition, for the CCA, we added the growth rate along with the physiological parameters to link this life-history trait with physiology.

Results

At the start of F-0, the overall survival rate was 88.4% and none of the variables had significant effects on survival (latitude: Df = 1, $X^2 = 3.47$, $p = 0.063$; temperature: Df = 1, $X^2 = 0.07$, $p = 0.789$; latitude × temperature: Df = 1, $X^2 = 2.58$, $p = 0.108$) but the survival tended to be lower at central latitudes. No larvae died during the 5-day exposure period to the predator cues in F-0.

Effects of native and invasive predator cues on growth rate, and how these depend on temperature and prey latitude

For growth rate, the overall model detected a significant effect of the covariate thermal exposure (estimate = -1.2e-04; SE = 5.7e-05) and of latitude with central-latitude larvae growing faster than high-latitude larvae (Least Square Mean [LSM] ± $\text{SE}_{\text{central latitude}} = 0.013 \pm 7.2 \times 10^{-4}$; $\text{LSM}_{\text{high latitude}} = 0.009 \pm 6.0 \times 10^{-4}$) (Table 1). Next, we performed separate analyses for each crayfish predator vs. control separately. First, we ran a model selection analysis to identify the average model; results are shown in Suppl. material 1: table S3. For the native noble and the invasive signal crayfish, both occurring at the two prey latitudes, almost similar models were selected that showed similar response patterns which were not affected by temperature (Suppl. material 1: table S4). The significant latitude × predator cues interaction indicated that while central-latitude larvae increased growth rate in response to predator cues of these two crayfish species, the high-latitude larvae showed the opposite response (Suppl. material 4; Fig. 2A, B). For the invasive spiny-cheek crayfish, which only occurs at the central latitude, the average model identified a significant effect of sex; with males growing faster ($\text{LSM}_{\text{male}} = 0.011 \pm 0.00$; $\text{LSM}_{\text{female}} = 0.008 \pm 0.00$) and of predator × latitude × sex, here the growth rate did not increase in response to spiny-cheek cues in central-latitude larvae, yet also decreased in high-latitude females (Suppl. material 1: table S4; Fig. 2C). Also, the growth responses to spiny-cheek cues were not affected by temperature (Suppl. material 1: table S4).

Table 1. Results of the GLMMs testing for the effects on growth rate, energy available (Ea), energy consumed (Ec) and cellular energy allocation (CEA) of sex (male vs. female), temperature (20 °C vs. 24 °C), predator (control vs. noble vs. signal vs. spiny-cheek crayfish), latitude (central vs. high latitude) and their interactions; thermal exposure was also added as a covariate and population as a random factor.

		Growth rate	**Physiological parameters**		
			Ea	**Ec**	**CEA**
Variables	**DF**	***p* (X^2)**	***p* (X^2)**	***p* (X^2)**	***p* (X^2)**
Thermal exposure	1	**0.036 (4.42)** *	**< 0.001 (25.2)** ***	**< 0.001 (25.0)** ***	0.378 (0.78)
Sex	1	0.079 (3.09) $^{(*)}$	0.191 (1.71)	0.052 (3.79) $^{(*)}$	0.449 (0.57)
Temperature	1	0.719 (0.13)	0.623 (0.24)	**< 0.001 (12.0)** ***	0.054 (3.72) $^{(*)}$
Predator	3	0.281 (3.82)	0.688 (1.48)	**0.002 (15.2)** **	0.054 (7.63) $^{(*)}$
Latitude	1	**0.032 (4.62)** *	**< 0.001 (33.2)** ***	0.572 (0.32)	**0.001 (10.8)** **
Predator × temperature	3	0.871 (0.71)	0.177 (4.92)	0.645 (1.66)	0.073 (6.98) $^{(*)}$
Temperature × latitude	1	0.532 (0.39)	0.242 (1.37)	**< 0.001 (14.5)** ***	0.062 (3.48) $^{(*)}$
Predator × latitude	3	0.170 (5.02)	0.782 (1.08)	0.185 (4.83)	0.267 (3.94)
Predator × temperature × latitude	3	0.972 (0.23)	0.408 (2.90)	0.504 (2.34)	**0.024 (9.43)** *
Random factor		***p* (X^2)**	***p* (X^2)**	***p* (X^2)**	***p* (X^2)**
Population	1	1.00 (0.00)	**< 0.001 (30.2)** ***	**< 0.001 (53.6)** ***	**0.008 (7.12)** **

Table shows degree of freedom (DF), p-values and the Wald X^2 in parentheses. Significance is indicated in bold by ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; $^{(*)}$ $p < 0.1$. Significance of the random factor was tested using likelihood ratio test.

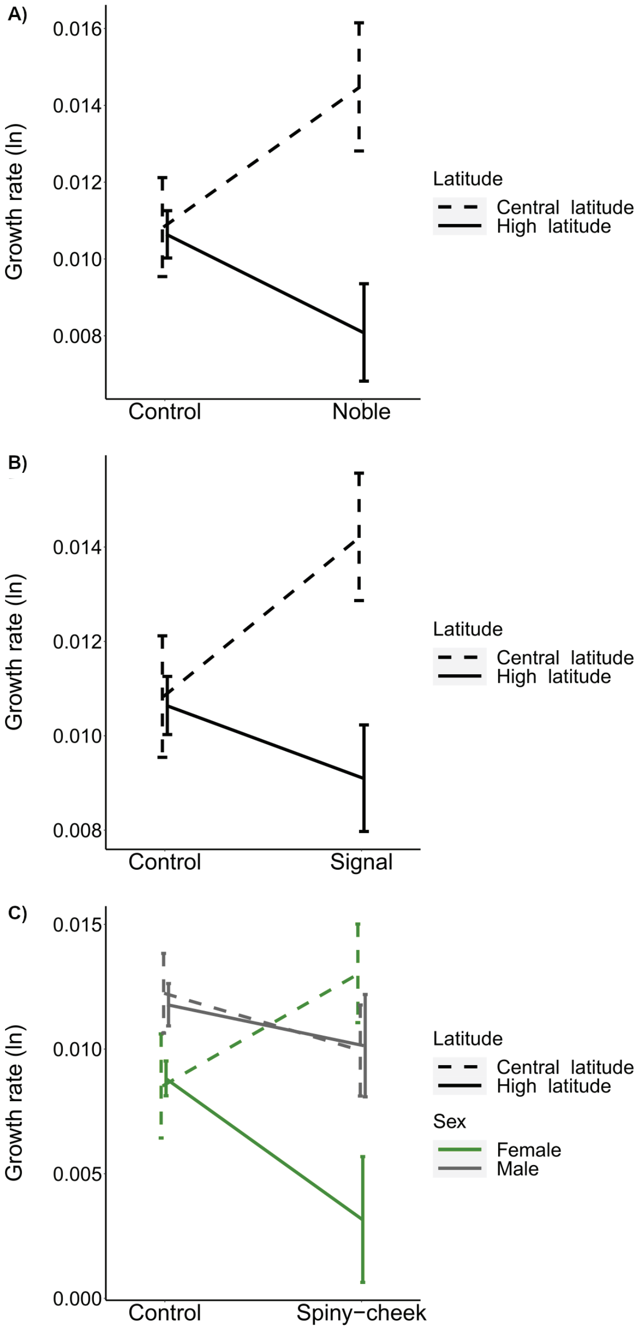

Figure 2. Effects of the predator cues on growth rate of the damselfly larvae of both latitudes for the three crayfish species **A** noble predator **B** signal predator, and **C** spiny-cheek predator. For the latter predator sex-specific effects were illustrated. Error bars indicate one standard error.

Effects of native and invasive predator cues on the bio-energetic response variables, and how these depend on temperature and prey latitude

The predator cues and temperature did not affect the energy available (Ea) (Table 1). Instead, Ea differed between latitudes and was higher in central-latitude prey ($LSM_{central} = 150 \pm 2.40$; $LSM_{high} = 118 \pm 2.11$) (Table 1). The energy consumed (Ec) was affected by the predator cues ($LSM_{control} = 20.6 \pm 0.4$; $LSM_{noble} = 19.2 \pm 0.4$; $LSM_{signal} = 18.8 \pm 0.4$; $LSM_{spiny\text{-}cheek} = 19.9 \pm 0.4$) with predation risk lowering the Ec for signal cues compared to control (df = 311, t = 3.21, p = 0.008) and, as a trend, for noble crayfish cues (df = 311, t = 2.40, p = 0.08), but not for spiny-cheek cues (df = 311, t = 1.29, p = 0.57). The Ec was lower at 24 °C than at 20 °C (LSM $_{20\,°C}$ = 19.8 ± 0.292; LSM $_{24\,°C}$ = 19.5 ± 0.27). The effect of the predator cues on Ec did not depend on temperature (Table 1). The significant interaction temperature × latitude indicated that more energy was consumed at central latitudes at 24 °C (Suppl. material 1: fig. S2). For both Ea and Ec, the analysis revealed a significant effect of population (Table 1), explaining 8.9% and 15.6% of the variance respectively.

For the cellular energy allocation (CEA), there was a significant effect of latitude with higher CEA at the central latitude (LSM $_{\mathrm{central}}$ = 7.39 ± 0.12; LSM $_{\mathrm{high}}$ = 6.30 ± 0.10) and a significant three-way predator cue × temperature × latitude interaction (Table 1, Fig. 3). The three way-interaction showed that exposure to cues from the native noble and, as a trend, from the invasive signal caused at both latitudes a higher CEA at 24 °C than at 20 °C (Suppl. material 4; Fig. 3A, B). While cues from the invasive spiny-cheek crayfish cues also only caused an increase in CEA at 24 °C, but not at 20 °C, this was only the case in central-latitude larvae while there was no effect of these cues in high-latitude larvae (Fig. 3C). Finally, the effect of population was also significant for CEA explaining 2.9% of the variance.

Effects of temperature and latitude on ETS and energy storage for each predator treatment

The MANOVAs testing for the effects of temperature and latitude for each predator vs. control treatment on the four physiological parameters used to calculate CEA: ETS, and fat, sugar and protein contents are presented in details in Suppl. material 1: file S2.

The analyses performed on the noble vs. control and signal vs. control treatment revealed similar patterns with significant effects of latitude, predator and of the interaction temperature × latitude (Suppl. material 1: table S6). At high latitude, combined or not with 24 °C, there were lower fat and sugar contents. In the presence of the predator cue, we observed a lower sugar content and growth rate.

The pattern was different for the spiny-cheek vs. control treatment with a significant three-way interaction predator × latitude × temperature cue (Suppl. material 1: table S6). At high latitude, in the presence of the spiny-cheek crayfish cue and at 24 °C, we observed lower fat and sugar contents and higher ETS activity.

Discussion

We investigated to what extent native and invasive crayfish species cause different indirect effects on prey growth and physiology, and to what extent these differences between predator types were further altered by temperature and prey latitude of origin. All three crayfish species, when analysed together, caused indirect effects on physiology (Ec and CEA) and interacted or not with other factors, providing weak support for the NPH. Indeed, the native noble and invasive signal crayfish, which both co-occur with the prey at the two latitudes, caused similar response patterns for growth and bio-energetics, while the invasive spiny-cheek crayfish caused somewhat different response patterns relative to both other crayfish species that depended on latitude. The weak support for the NPH was not further modulated by temperature or prey latitude of origin. While indirect effects on growth rate were consistent across temperatures, yet strongly latitude-dependent across all three crayfish species, indirect effects on physiology were only detected at 24 °C and were less latitude-dependent.

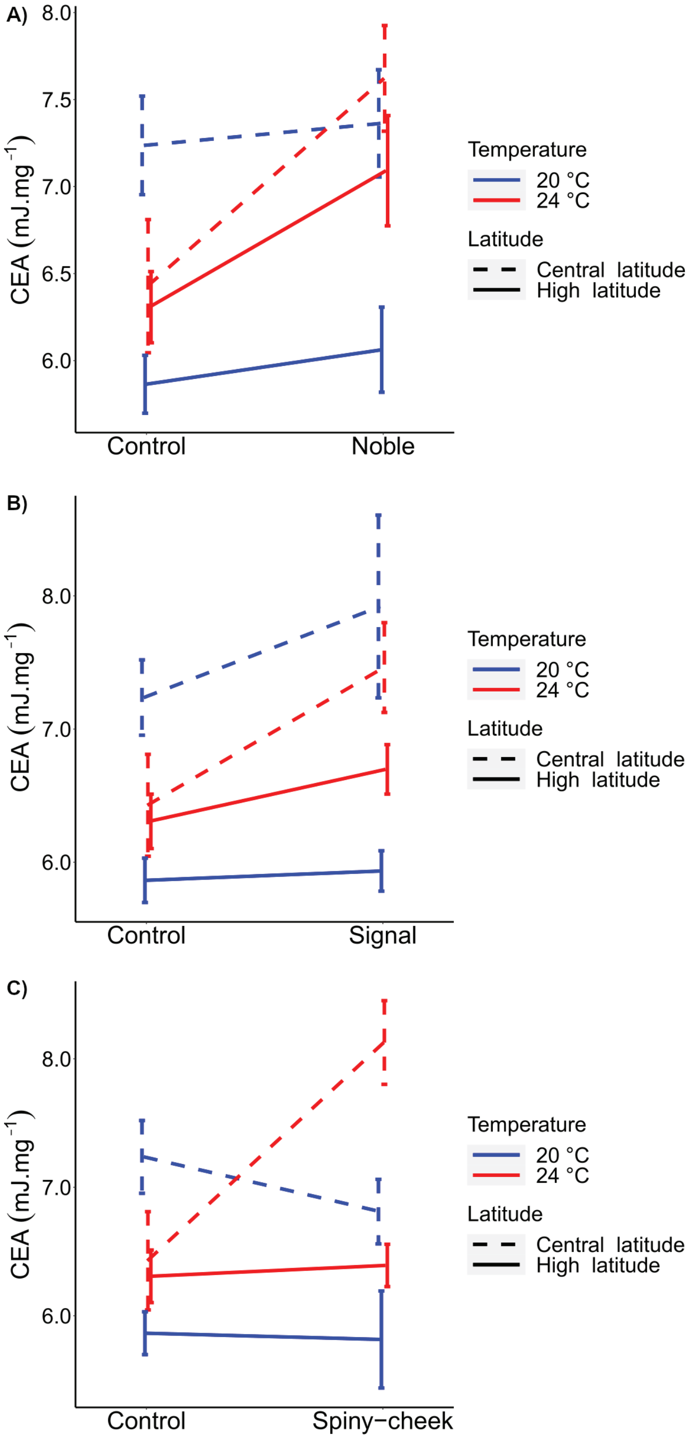

Figure 3. Effects of the predator cues and temperature on the cellular energy allocation (CEA) of the damselfly larvae of both latitudes for the three crayfish species **A** noble predator **B** signal predator, and **C** spiny-cheek predator. Error bars indicate one standard error.

Combined effects of predator, latitude and temperature on growth rate

In general, our growth results did not provide strong support for the NPH. When crayfish species are analysed together or separately, no clear or consistent differences in larval growth rate were found when exposed to native versus invasive predator species. Our results contrasted with previous studies in the damselfly *I. elegans* which found the opposite of the NPH with an increase in egg developmental time when exposed separately to invasive signal and spiny-cheek crayfish vs. native perch fish (Antoł and Sniegula 2021) and in egg developmental time and mass at emergence when exposed to invasive signal crayfish vs. native perch in central-latitude populations (Amer et al. 2024). Another study found increased negative effects on egg and larval survival, egg development time and larval mass and fat content when treated with alien danube and invasive alien signal vs. native noble crayfish cues (Sniegula et al. 2025). Also, weak support for the NPH was found in other prey species, for instance, in *Daphnia mendotae* no difference in adaptive behavioural responses was detected to various invasive and native predators (Bourdeau et al. 2013). To explain deviations from the NPH, it has been proposed that being exposed to other crayfish predators in nature (e.g., noble and signal crayfish present at high latitudes) might enable prey to recognize predator cues released from an unestablished, novel invasive predator (spiny-cheek crayfish here) and trigger similar responses (Anton et al. 2020). In that sense, the three crayfish species used in the current study probably shared some similarities in term of chemical cues released in the environment. Indeed, not established invasive predators may produce similar kairomones in terms of chemical composition as established invasive or native predators, which is more likely with a higher degree of phylogenetic relatedness (Sih et al. 2010). Nevertheless, there is conflicting evidence for this idea. For example, there was no relationship between taxonomic distance of various invasive predators belonging to different clades and the behavioural response of their *Daphnia* prey (Bourdeau et al. 2013). In our study, noble and signal crayfish are phylogenetically close and belong to the same family (Owen et al. 2015). Chemical cues from these two crayfish species triggered similar latitude-specific responses in the prey. The spiny-cheek crayfish belongs to a different family and it is unclear to what extent kairomones produced by this crayfish are similar to those produced by the other crayfish species. Furthermore, we did not report the sex of the collected crayfishes and it is unclear to what extent male and female crayfishes differ in the nature and quantity of kairomones produced. As we controlled only for crayfish biomass, some variation in term of quantity or quality of kairomones between aquariums cannot be excluded with potential effects on the larval response. Nevertheless, as the prey only had chemical cues to rely on, the observation that central-latitude prey did not change their growth rate when exposed to spiny-cheek cues but did it when exposed to cues from the other crayfish species suggests differences in the chemical composition of these cues. Hence, despite the characteristics of freshwater ecosystems and small waterbodies that tend to favour isolation and naivete (Cox and Lima 2006; Anton et al. 2020), we provided evidence that recognition of novel alien predators may occur and was probably due to some degrees of phylogenetic relatedness between native and invasive predators.

Contrary to our expectations, temperature had no significant effects on the larval growth response to predators (Stoks et al. 2012; Amer et al. 2024), hence also not on the weak NPH patterns. A previous study also showed that larvae had similar growth rate at 20 °C and 24 °C when exposed to the invasive spiny-cheek crayfish cues, but an increase in growth rate under crayfish stress was observed at 28 °C (Palomar et al. 2023). Therefore, the effects of temperature on this particular predator-prey interaction may be manifested only under higher temperatures.

Despite some clear latitude-specific patterns in the prey growth responses, these had no clear and consistent effect on the weak NPH patterns. Our results confirmed the general trend that central-latitude populations grew faster due to the higher voltinism linked to the longer growth season at the central latitude (Śniegula et al. 2012; Dinh Van et al. 2014). These latitudinal differences in growth rate mainly persisted under predation risk, while both prey latitudes responded in opposite directions. For high-latitude damselflies, that are less time-constrained, we found a consistent growth reduction across the three crayfish species probably because decreased growth can be adaptive to avoid predation (Stoks et al. 2005b) and can be compensated by more time available for reaching the final size prior emergence relative to the central-latitude populations. Even though the spiny-cheek crayfish has not been yet reported at high latitudes, damselflies were capable of producing a growth response, which was similar to their response to cues from the native predator at least for female damselflies (sex-specific effects described below). For central-latitude damselflies, however, the noble and signal crayfish species caused a growth acceleration, while the spiny-cheek crayfish triggered no significant growth response. A growth acceleration in response to predation risk has been observed before for the study species (Stoks et al. 2012), and may be adaptive by reducing the duration of exposure to aquatic predators especially in time-constrained prey populations. One possible reason why such growth acceleration was not present in response to spiny-cheek cues is that this species is more abundant in southern Poland than the two other species studied and we may hypothesize that these differences in selective pressures might have been selected against a risky growth acceleration.

Interestingly, only high-latitude female larvae responded with a growth reduction when exposed to the spiny-cheek crayfish cues. Sex-specific responses to predation risk have been demonstrated in previous studies. For example, predation risk caused a decrease in development time in female but not male mosquitoes (Fontana-Bria et al. 2017) and a greater metabolic rate in male but not female crickets (Lagos and Herberstein 2017). In general, sex-specific effects are more pronounced in species with strong sexual dimorphism and in protandrous species where males and females have different growth rates which is often the case in damselflies (Corbet 1999). In addition, females may be more sensitive to some stressors, e.g. food stress, as they require more energy for their development to reach a higher body mass compared to males (Teder and Kaasik 2023).

Combined effects of predator, latitude and temperature on physiology

Bio-energetic response patterns to temperature and predation risk only partly matched our observations for growth rate, indicating a partial decoupling between life history and physiology. As was the case for growth rate, we observed a distinct physiological response when exposed to the spiny-cheek crayfish cues whereas noble and signal crayfishes tended to cause more similar effects, again providing weak support for the NPH. In addition, patterns may be also population-dependent as previously demonstrated in *Lestes* species at the physiological level with variation in assimilation efficiency between permanent and temporary ponds observed under predation risk (Stoks and McPeek 2003). In our study, despite significant effects of population on physiology, we lack enough replicates at the population level (only two ponds per latitude) to further explore such interactions at the local scale but this opens avenues for further investigations.

For the CEA, the response to native and invasive predators depended on both temperature and latitude (significant three-way interaction), revealing more complex patterns than initially predicted on how both factors would affect the predator-prey and NPH patterns. Our results showed that CEA was increased when prey were exposed to predator cues but only at 24 °C, supporting our prediction that an increase in temperature would increase the indirect effects of predators as perceived predation risk is likely higher. Yet, and in contrast to our NPH prediction, no overall clear distinction was observed in this response between native and invasive predator species. Nevertheless, there was some support for the prediction that the latitude-specific evolutionary history with predators played a role, as the increase in CEA under predation risk when combined with 24 °C was for spiny-cheek crayfish cues only present in prey of the central latitude and not of the high latitude. This is as expected by the NPH as the spiny-cheek crayfish only occurs at the central but not the high latitude. In general, higher CEA values indicate more energy being available for growth and reproduction which was consistent with the overall faster growth rate and CEA under predation risk in central-latitude prey. This result contrasted with a tendency for a lower CEA under predation risk in larvae of the confamiliar damselfly *E. cyathigerum* (Van Dievel et al. 2019). However, in that case the authors exposed larvae to the dragonfly predator cues for a longer period of time (9 days). In such a case, predator exposition may reveal some trade-offs, e.g. between growth and defence mechanisms (Van Dievel et al. 2019).

Our results revealed that variation in CEA was mainly due to variation in Ec rather than in Ea. Indeed, we found a significant increase of CEA in response to predator cues (at 24 °C) which was accompanied by a reduction of Ec (manifested by a lower ETS activity), especially when exposed to noble and spiny-cheek crayfish cues. For Ea, we observed a considerable reduction in sugar content in the presence of both native and invasive predator cues and smaller effects on fat and protein contents, which together, however, did not translate in lower Ea under predation risk. Previous studies conducted on *E. cyathigerum* also reported a sugar reduction but this combined with a higher metabolic rate in the presence of a predator cue suggesting an escape strategy and the mobilization of important energy resources in prey (Janssens et al. 2015; Van Dievel et al. 2016). In our study, a lower metabolic rate under predation risk may reflect the often observed reduced foraging activity in prey (Krams et al. 2013) e.g. in damselflies (Kohler and McPeek 1989), with sugar reserves being used as a direct source of energy to maintain growth and vital functions. We hypothesise that fat and proteins may be used as sources of energy if the stressor persists for a longer period of time. Altogether, these results indicate some shared responses in term of energy use in response to predator cues (especially noble and signal crayfish), independently of their evolutionary histories with their prey. Some differences arose from the spiny-cheek crayfish which triggered, as for growth rate, some differential effects that were here jointly temperature- and latitude-dependent. The combination of this specific predator cue and temperature may represent particularly stressful conditions for central-latitude damselflies and maintaining high metabolic activity may be especially costly, as no growth acceleration was found under this predator cue treatment.

Conclusions

There is increasing concern on the effects of biological invasions and that these may be stronger under human-induced global warming. Overall, our results provide only partial support for the NPH. Indeed, the responses to the native predator were very similar for one of the invasive predators (the signal crayfish), but differed for the other invasive predator (the spiny-cheek crayfish). We found some support for the idea that the prey latitude of origin may shape the impact of invasive predators as central- and high-latitude prey responded differently to both invasive predators, yet this latitude-specific response pattern was shared between the native predator and one of the invasive predators. Furthermore, high-latitude prey populations were able to recognize the spiny-cheek crayfish which is currently absent at this latitude. Our results did show stronger indirect effects imposed by the two invasive crayfish (signal and spiny-cheek crayfish) on the bio-energetic variables at 24 °C, supporting the concern that global change factors may magnify the impact of invasive predators. Yet, our results also indicated that the indirect effects at 24 °C were not stronger when exposed to invasive vs. native crayfish predators. Taken together, while the indirect effects when exposed to invasive crayfishes may show similarities to the ones imposed by native crayfish species, our results indicate they may cause additional stress on the local prey populations, especially at high-latitudes at 24 °C.

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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, RS. Formal analysis: GW. Funding acquisition: SS. Methodology: NRA, AA, SS. Writing - original draft: GW. Writing - review and editing: RS, SS, NRA, GW, AA.

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Data availability

Data used in the manuscript are included in Suppl. material 3.

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Supplementary material 1

Additional information

Authors: Guillaume Wos, Nermeen R. Amer, Andrzej Antoł, Robby Stoks, Szymon Sniegula

Data type: docx

Explanation note: **file S1.** Physiological analysis - Methods. **file S2.** Effects of temperature and latitude on ETS and energy storage for each predator treatment – Results. **fig. S1.** A) Monthly and B) weekly temperatures for each Polish (PL; central latitude) and Swedish (SW; high latitude). **fig. S2.** Plot showing the significant interaction temperature × latitude on cellular energy consumed (Ec). **table S1.** GPS coordinates of the sampled ponds for Ischnura elegans. **table S3.** Results of the model selection analysis for growth rate for each predator species vs control treatment. **table S4.** Results of GLMM testing for the effects on growth rate final (GR Final). **table S6.** Results of the targeted Multivariate Analyses of Variance (MANOVA) per crayfish species showing the effects. **table S7.** Results of the canonical constrained analysis. **table S8.** Results of the canonical constrained analysis (CCA).

Link: https://doi.org/10.3897/neobiota.98.141133.suppl1

Supplementary material 2

Effects of the significant predictors detected by MANOVA

Authors: Guillaume Wos, Nermeen R. Amer, Andrzej Antoł, Robby Stoks, Szymon Sniegula

Data type: pdf

Explanation note: Effects of the significant predictors detected by MANOVA (Suppl. material 1: table S6) on physiological parameters for each comparison A) noble vs control, B) signal vs control and C) spiny-cheek vs control treatment. Growth rate (GR) was added on the plot along with the physiological parameters. Scores of each trait and variable are shown in Suppl. material 1: table S8. Abbreviations: high latitude (HL) and spiny-cheek crayfish (S-C).

Link: https://doi.org/10.3897/neobiota.98.141133.suppl2

Supplementary material 3

Dataset used in the study

Authors: Guillaume Wos, Nermeen R. Amer, Andrzej Antoł, Robby Stoks, Szymon Sniegula

Data type: xlsx

Explanation note: 1) Dataset including growth rate, electron transport activity (ETS), and fat, sugar and protein contents for each individual. 2) Sample size for each latitude, treatment and sex.

Link: https://doi.org/10.3897/neobiota.98.141133.suppl3

Supplementary material 4

Pairwise comparisons

Authors: Guillaume Wos, Nermeen R. Amer, Andrzej Antoł, Robby Stoks, Szymon Sniegula

Data type: xlsx

Explanation note: Pairwise comparison testing for the difference in growth rate and cellular energy allocation (CEA) between the two latitudes and temperatures in the different predator treatments. Significance is indicated in bold.

Link: https://doi.org/10.3897/neobiota.98.141133.suppl4

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Research Article

Increasing breeding host range and fast spread across Uruguay reveals the invasion potential of *Euwallacea fornicatus* (Coleoptera, Scolytinae) in South America

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Abstract

The invasive ambrosia beetle *Euwallacea fornicatus* (Eichhoff, 1868) poses a significant threat to forests *sensu latu* in South America. Uruguay marked the third regional record since 2022, following infestations in Brazil and Argentina. The pest's distribution now spans ~ 3,500 km of coastline, highlighting its adaptability to diverse climates and the vulnerability of urban ecosystems. Currently, two infestations started in Uruguay in two foci distant 500 km of each other: Rivera, a northern city bordering with Brazil and an established beetle population in Montevideo, the capital and a port city in the southern region of the country. The infestation in Montevideo initially mirrors those in the city of Buenos Aires, Argentina, with low attack densities (< 30 attacks/tree) concentrated at lower trunk heights (< 4 m). The key host species include *Acer negundo* L., *Casuarina cunninghamiana* Miq. and *Platanus × acerifolia* (Aiton) Willd., indicating an affinity for common urban trees in the Southern Cone. This pest is known to attack 602 plant species. However, our host plant survey recorded fourteen new host plant species, with nine new breeding hosts. While tree mortality is not yet evident, gallery excavation and symbiotic fungal activity threaten tree health and tend to escalate management costs. Molecular analyses confirm the presence of a single haplotype within *E. fornicatus* species, which is phylogenetically close to Argentine, Brazilian, Chinese and European populations. The rapid spread across Uruguay along with the increasing number of hosts with breeding potential, both exotic and native in Argentina and Uruguay, highlights the significant invasion threat this species poses for South America. It is crucial to implement coordinated supranational management strategies without delay. Considering the size of the populations, they should include eradication efforts using mechanical and chemical means, followed by continuous monitoring to prevent re-emergence or re-introduction of propagules from neighbouring countries.

Resumen

El escarabajo de ambrosía invasor *Euwallacea fornicatus* (Eichhoff, 1868) representa una amenaza significativa para los bosques *sensu latu* de América del Sur. Uruguay ha sido el tercer registro regional desde el año 2022, tras las infestaciones de Brasil y Argentina. La distribución de esta plaga abarca aproximadamente 3.500 km de costa, lo que destaca su adaptabilidad a climas diversos y la vulnerabilidad de los ecosistemas urbanos. Actualmente, las infestaciones en Uruguay comenzaron en dos focos distantes por 500 km: Rivera, una ciudad norteña y fronteriza con Brasil, y una población de la plaga establecida en Montevideo, ciudad al sur, portuaria y capital del país. Los patrones de infestación en Montevideo reflejan los observados al inicio en la ciudad de Buenos Aires, Argentina, con densidades de ataque bajas (< 30 ataques/árbol) concentradas en las partes bajas de los troncos (<4 m). Las especies hospedantes clave incluyen *Acer negundo* L., *Casuarina cunninghamiana* Miq., y *Platanus* × *acerifolia* (Aiton) Willd., lo que indica una afinidad por árboles urbanos comunes para el Cono Sur. Esta plaga ataca a 602 especies de plantas. Sin embargo, el presente trabajo ha podido registrar catorce nuevas especies de plantas hospedadoras, con nueve hospedantes reproductivos nuevos. Si bien aún no se observa mortalidad de árboles, la excavación de galerías y la actividad fúngica simbiótica amenazan la salud de los ejemplares arbóreos y tienden a incrementar los costos de manejo. Los análisis moleculares confirman la presencia de un único haplotipo dentro de la especie *E. fornicatus*, filogenéticamente cercano a las poblaciones argentinas, brasileñas, chinas y europeas. La rápida expansión de esta especie en Uruguay, sumado al incremento de hospedantes con potencial reproductivo, tanto exóticos como nativos en Argentina y Uruguay, subraya la grave amenaza de invasión que representa para América del Sur. En consecuencia, resulta fundamental la implementación inmediata de estrategias de manejo supranacional coordinadas para mitigar su impacto. Considerando el tamaño poblacional de la plaga, deberían incluir esfuerzos de erradicación utilizando medios mecánicos y químicos, seguidos de un monitoreo continuo para prevenir la reemergencia o reintroducción de inóculos desde países vecinos.

Key words: Ambrosia beetle, host plants, invasive species, pest, PSHB, Scolytinae, Southern Cone, America

Introduction

Invasive species pose critical threats to forest ecosystems globally, disrupting biodiversity, impacting native species and altering ecosystem processes (Mack et al. 2000; Pimentel et al. 2005; Vila et al. 2011). The increasing international trade has amplified the spread of invasive species across continents, facilitating the movement and establishment of highly adaptive organisms such as ambrosia beetles from the tribe Xyleborini (Haack 2006; Aukema et al. 2010). This tribe includes some of the most successful invaders, characterised by their small size, fungus-cultivating behaviour and ability to thrive on a wide range of host trees (Jordal et al. 2001). Additionally, their reliance on sib-mating enhances their invasive potential, allowing a single or a few individuals to establish a population in a new region (Brockerhoff and Liebhold 2017; Hughes et al. 2017).

Within this group, *Euwallacea fornicatus* (Eichhoff, 1868) commonly known as the polyphagous shot hole borer (PSHB) and part of the *Euwallacea fornicatus* species complex, comprises several species with slight morphological variations that often require molecular data for accurate identification (Stouthamer et al. 2017; Gomez et al. 2018). Originally native to Asia, members of this complex are notorious for infesting both urban and natural forests and have impacted economically valuable tree species such as avocado and timber trees (Gomez et al. 2019; Ruzzier et al. 2023; Goldarazena et al. 2025). Their ability to adapt to non-native regions such as Australia, Israel, Spain, South Africa and the United States, highlights their adaptability and underscores the need for monitoring and controlling these pests in newly-affected areas (Paap et al. 2018; Schuler et al. 2023).

Reports of *E. fornicatus sensu stricto* in South America have been sparse and often fraught with identification challenges. However, recent studies employing DNA data have confirmed its presence in Argentina and Brazil (Fig. 1). In Argentina, it causes damage to several species of urban trees in Buenos Aires such as *Populus deltoides* W. Bartram ex Marshall (Salicaceae) and *Platanus × acerifolia* (Aiton) Willd. (Platanaceae) (Ceriani-Nakamurakare et al. 2023), whereas in Brazil, it has been reported in five States, mainly damaging *Persea americana* Mill. (Lauraceae) and *Khaya grandifoliola* C.DC. (Meliaceae), as part of a nationwide survey of bark and ambrosia beetles (Covre et al. 2024). Despite extensive research in other regions, there remains a critical knowledge gap regarding host-plant relationship between *E. fornicatus* and South American native species. This deficiency hinders our understanding of the pest's impact on regional biodiversity and its invasion potential. A comprehensive host-plant assessment is therefore essential to elucidate the ecological and economic risks that this forest pest poses to South American ecosystems.

Here, for the first time, we report *E. fornicatus* in Uruguay on the basis of morphological and molecular analyses of specimens collected from urban trees in the southern and the northern regions of the country. Additionally, we expand the record of attacked native and exotic plant species that are frequently employed in urban forests along the South American region, thus providing critical information on distribution and confirmed host preferences.

Materials and methods

In March 2023, unusual symptoms on an *Acer japonicum* Thunb. (Aceraceae) known as Japanese maple were detected within the Montevideo Japanese Garden *Hei Sei En*, Uruguay (34°51'S, 56°12'W). In October 2024, the same symptoms on a decaying urban tree from *Ficus* genus (Moraceae) were reported in Rivera City, Uruguay (30°54'S, 55°30'W) (Fig. 1).

The symptoms consisted of multiple entry holes (approximately 1 mm in diameter) distributed across the trunk and main branches, with visible sawdust accumulation and sap exudation. Following initial detection, wood samples containing active galleries were collected from the affected tree. Live adult beetles were collected from the galleries and immediately preserved in absolute ethanol at -18 °C for subsequent morphological and molecular analyses. Voucher specimens were deposited in the Entomological Collection of Facultad de Agronomía, Universidad de La República. Additionally, a targeted field survey was conducted to determine the extent of infestation and distribution within key areas, including the ports, Japanese Garden, Botanical Garden and linear tree plantings featuring non-native tree species, such as *Platanus × acerifolia*, *Acer japonicum*, *Acer negundo* L. (Aceraceae), *Casuarina cunninghamiana* Miq. (Casuarinaceae) and *Populus* spp. L. (Salicaceae).

Taxonomic identification was based on direct observation using a Leica M205A stereomicroscope. Elytra and pronotum lengths were measured from base to apex in lateral view, diagnostic characters for the *Euwallacea fornicatus* species complex (Gomez et al. 2018; Smith et al. 2019). Additionally, DNA extraction was conducted following the methods of Gomez et al. (2018). The mitochondrial gene cytochrome oxidase c subunit I (COI) was amplified through PCR using the primer pair COI_1455b and COI_r750 (Smith and Cognato 2014). The PCR and thermocycling conditions were as reported in Gomez et al. (2018) and the amplified products were sequenced by Macrogen (Korea). The sequencing data were assembled, trimmed and compared with the DNA sequence alignment in Ceriani-Nakamurakare et al. (2023) with the addition of Brazilian data (Covre et al. 2024), which represent the most recent and comprehensive alignments available

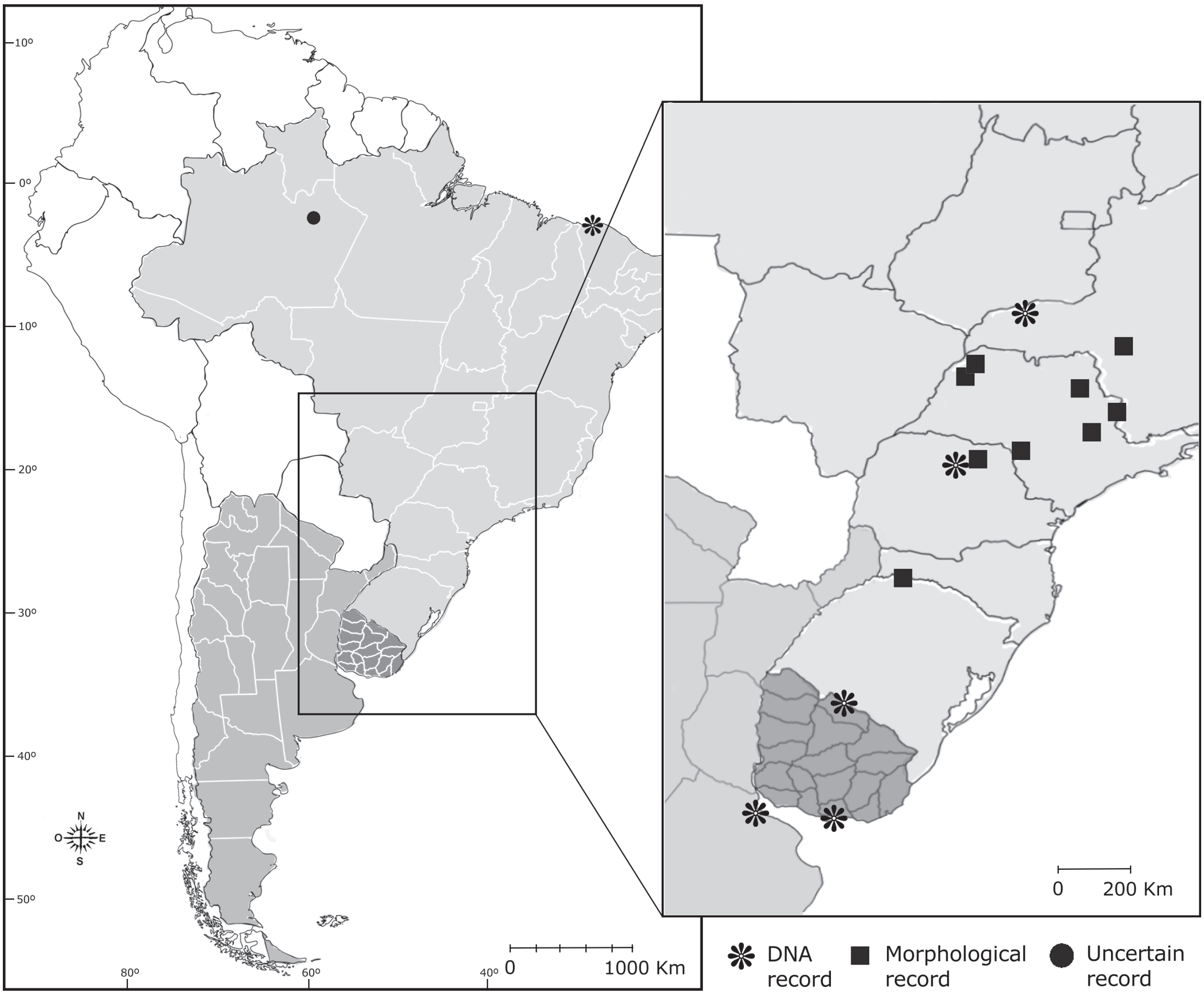

Figure 1. South American geographic distribution of *Euwallacea fornicatus*. an Asterisk samples from which DNA was studied; black circle uncertain historical record (Wood 2007); and black rectangle records confirmed through non-molecular methods.

for the *E. fornicatus* species complex in South America. The COI sequence was deposited in GenBank under accession number (PQ667063) for both Montevideo and Rivera specimens (i.e. 100% identity).

A survey of the host plant range of *E. fornicatus* was conducted from 2024 to 2025 at the Carlos Thays Botanical Garden, Buenos Aires, Argentina. Sampling efforts focused on plant stems and branches up to 2.5 m above the ground. Attacked plant tissues were collected and analysed in the laboratory to confirm the identity of the beetle and to determine its reproductive status by assessing the presence of larvae or pupae. When plant material could not be obtained to verify direct evidence of breeding, larval sawdust and emerging adults were used as alternative indicators. The novelty status of each species was assessed using EPPO (2025). Suppl. material 1 compiles the importance of each plant species, categorised into six categories based on its primary relevance: Academic (A), representing its value for research and education; Consumable (C), indicating its use in food, medicinal or pharmaceutical contexts; Symbolic (S), for religious, cultural or spiritual significance; Material (M), related to industrial or commercial applications; Ornamental (O), referring to its aesthetic or landscaping uses; and Ecological (E), for roles in conservation, ecosystem functions or niche building. Species with undocumented or poorly understood uses were classified as Undefined (U). Additionally, the status of each plant species within the Argentinean and Uruguayan flora was determined as native or exotic and the conservation status was determined following IUCN Categories and Criteria (IUCN 2025) i.e. species were classified as Not Evaluated (NE), Data Deficient (DD), Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CR) or Extinct in the Wild (EW) based on available risk assessments.

Results

The number of attacks per individual tree in affected areas was fewer than thirty for the species *A. negundo*, *C. cunninghamiana* and *P.* × *acerifolia*. The symptoms of infestation are quite conspicuous (Fig. 2). In *A. negundo* and *P.* × *acerifolia*, re-infestation was observed, indicating an attack pattern consistent with previous findings (Ceriani-Nakamurakare et al. 2023).

Molecular analysis of the specimens from Montevideo and Rivera cities revealed high genetic similarity, with nearly 100% sequence identity to specimens from Argentina (GenBank: OR016051) and Brazil (GenBank: OR773079, OR773081). Both specimens from Uruguay exhibited 100% identity and only one base difference when compared to the sequence from Buenos Aires, Argentina and from Ceará and Paraná, Brazil. In contrast, two base differences were observed compared with the sequence from Minas Gerais, Brazil (GenBank: OR773080). Phylogenetic analyses indicated remarkable genetic conservation, with the sequence clustering closely with specimens previously collected in China and recently identified in European greenhouses (99.4–99.8% identity) (Fig. 3).

Our host plant survey revealed fourteen novel species for *E. fornicatus* (Table 1), including representatives from five newly-documented genera: *Araucaria* (Araucariaceae), *Geoffroea* (Fabaceae), *Blepharocalyx* and *Feijoa* (Myrtaceae) and *Gardenia* (Rubiaceae). In addition, it is the first record for Araucariaceae family and provides the first evidence of successful breeding in two previously reported non-reproductive host plants: *Ceiba speciosa* (A.St.-Hil., A.Juss. & Cambess.) Ravenna (Ebenaceae) and *Fraxinus excelsior* L. (Oleaceae). Amongst these novel associations, nine species were confirmed as breeding hosts from which six are native species (67%). The identified host plants play multiple ecological and economic functions (see Suppl. material 1) and show that twelve species have consumable uses (C), five possess ornamental value (O) and several species demonstrated additional importance categories. According to IUCN Red List Criteria, most species are classified as Least Concern (LC), while *Ficus aspera* G.Forst. (Moraceae) and *F. excelsior* are categorised as Near Threatened (NT) and *Brugmansia* × *candida* Pers. (Solanaceae) is listed as Extinct in the Wild (EW).

Discussion

The detection of *Euwallacea fornicatus* in Uruguay represents the third confirmed record in South America within a remarkably short period of time (2021–2024), suggesting rapid invasion and establishment throughout the region. The spatial epidemiological pattern in Uruguay suggests a multipoint invasion scenario with two distinct stages: one in the northern border city of Rivera and the other in the southern port city of Montevideo.

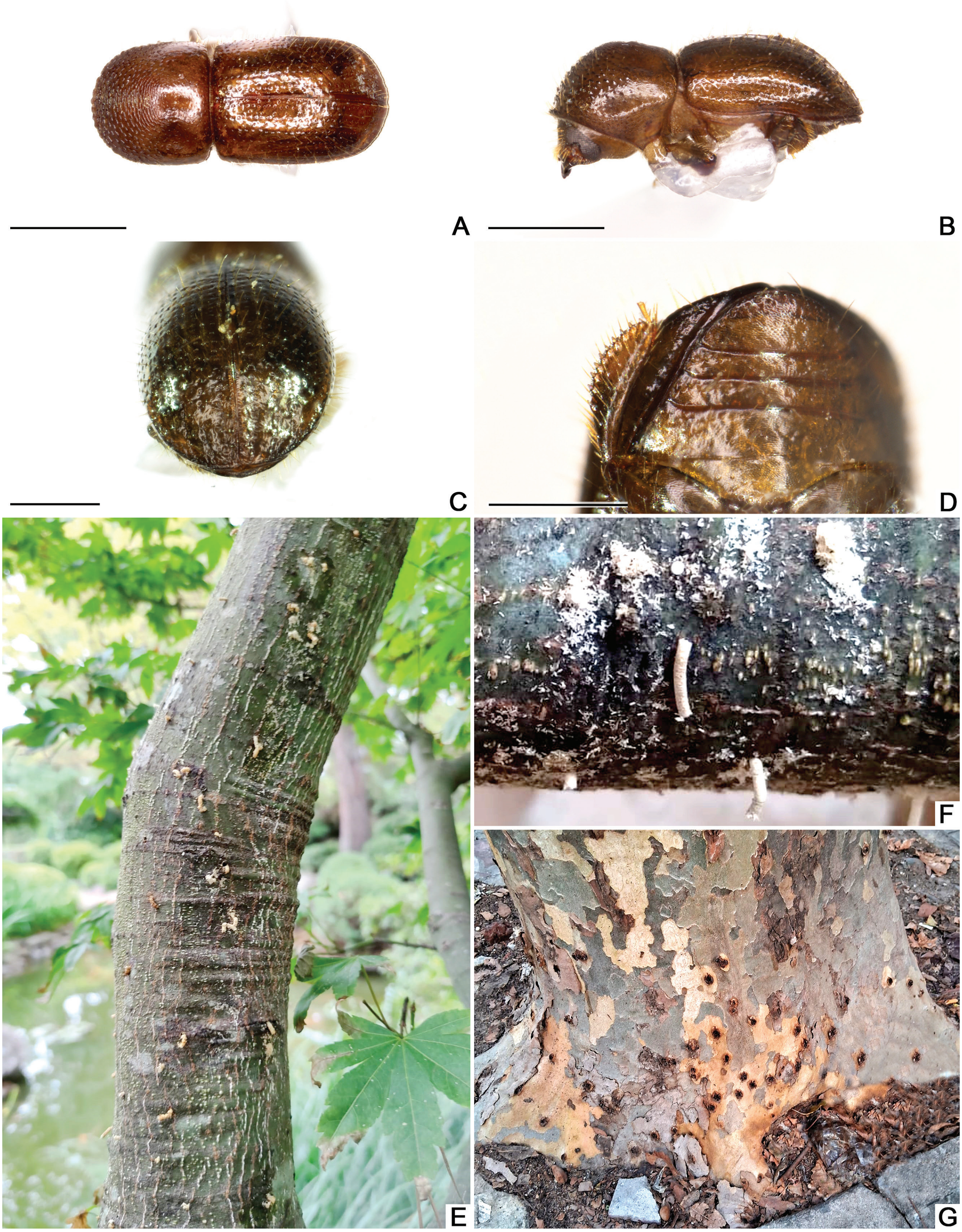

Figure 2. Morphology and symptoms of *Euwallacea fornicatus* **A–D** female specimen from Montevideo, Uruguay, showing dorsal, lateral, elytral declivity and protibial dentation views **E, F** symptoms caused by *E. fornicatus* on *Acer japonicum* **G** on *Platanus* × *acerifolia*. Scale bars: 1.00 mm (**A, B**); 0.50 mm (**C, D**). Pictures by M. Bollazzi.

Table 1. Host plant species attacked by *Euwallacea fornicatus* in Argentina, Brazil and Uruguay, including their novelty and breeding status.

Genus and Species	Author citation	Novelty status	Breeding Host	Native or Exotic (N/E)	Reference
Acacia mangium	Willd.	-	✓	E	Brazil[4]
Acer japonicum	Thunb.	-	✓	E	Argentina[3]
Acer negundo	L.	-	✓	E	Argentina[3]
Albizia julibrissin	Durazz.	-	✓	E	Argentina[3]
Araucaria columnaris	(J.R.Forst.) Hook.	new	X	E	present work
Bauhinia forficata	Link	new	✓	N	present work
Blepharocalyx salicifolius	(Kunth) O.Berg	new	X	N	present work
Brachychiton populneus	(Schott & Endl.) R.Br.	-	✓	E	Argentina[3]
Brugmansia × *candida*	Pers.	new	X	E	present work
Casuarina cunninghamiana	Miq.	-	✓	E	Argentina[3]
Ceiba speciosa	(A.St.-Hil., A.Juss. & Cambess.) Ravenna	known[1]	✓ (new)	N	present work
Celtis tala	Gillies ex Planch.	new	X	N	present work
Diospyros inconstans	Jacq.	new	✓	N	present work
Feijoa sellowiana	(O.Berg) O.Berg	new	X	N	present work
Ficus aspera	G.Forst.	new	✓	E	present work
Ficus religiosa	L.	new	X	E	present work
Fraxinus excelsior	L.	known[2]	✓ (new)	E	present work
Fraxinus sp.	Tourn. ex L.	-	unknown	E	Argentina[3]
Gardenia thunbergia	Thunb.	new	✓	E	present work
Geoffroea decorticans	(Gillies ex Hook. & Arn.) Burkart	new	✓	N	present work
Inga uraguensis	Hook. & Arn.	-	X	N	Argentina[3]
Inga vera	Willd.	-	X	E	Argentina[3]
Khaya grandifoliola	C.DC.	-	unknown	E	Brazil[4]
Morus alba	L.	-	✓	N	Argentina[3]
Myrsine laetevirens	(Mez) Arechav.	new	✓	N	present work
Neltuma caldenia	(Burkart) C.E.Hughes & G.P.Lewis	new	✓	N	present work
Persea americana	Mill.	-	✓	E	Brazil[4]
Platanus × *hispanica*	Mill. ex Münchh.	-	✓	E	Argentina[3]
Populus deltoides	W.Bartram ex Marshall	-	✓	E	Argentina[3]
Schinus longifolia	(Lindl.) Speg.	-	✓	N	Argentina[3]
Solanum granulosoleprosum	Dunal	-	✓	N	Argentina[3]
Solanum mauritianum	Scop.	-	✓	N	Brazil[4]
Tipuana tipu	(Benth.) Kuntze	-	X	N	Argentina[3]
Toona ciliata	M.Roem.	-	✓	E	Brazil[4]
Trichilia glabra	L.	new	X	E	present work

Notes. Recorded host plant species being attacked by *Euwallacea fornicatus* at the South American sentinel Botanical Garden "C. Thays" in Buenos Aires, Argentina. [1] Mendel et al. (2021). [2] van Rooyen et al. (2021). [3] Ceriani-Nakamurakare et al. (2023). [4] Covre et al. (2024).

In the northern region, the cross-border city of Rivera and its Brazilian counterpart, Santana do Livramento, form a binational urban agglomeration of approximately 250,000 inhabitants and a shared 12 km "dry border", i.e. a city street that serves as a geopolitical border. This dynamic border facilitates the daily movement of goods, people and, inadvertently, biological materials. Despite existing phytosanitary controls, limited resources prevent effective detection of inconspicuous pests such as *E. fornicatus*. Given its known presence in multiple nearby Brazilian localities (Covre et al. 2024), it is plausible that populations of this pest have

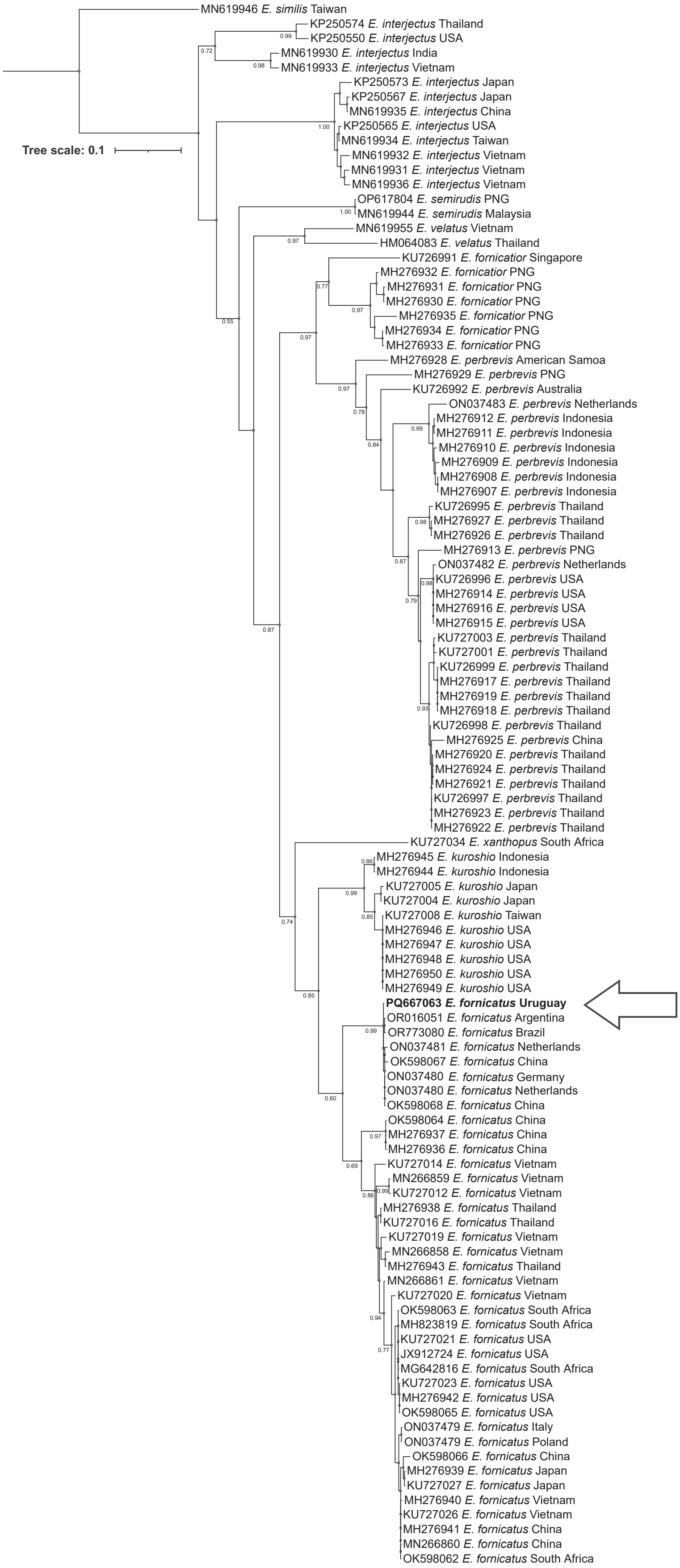

Figure 3. Maximum Likelihood phylogenetic tree of the *Euwallacea fornicatus* species complex generated using the general time-reversible model with bootstrap support values (1000 replicates). A distinctive arrow marks the phylogenetic position of the Uruguayan specimens.

expanded into the northern region of Uruguay through natural dispersion and/or accidental transport. Although Montevideo's population could potentially serve as an inoculum source for Rivera's invasion, the absence of symptoms on urban trees along National Route 5, the main connecting corridor, does not support this.

In contrast, the establishment of *E. fornicatus* in the southern city of Montevideo, detected in March 2023, suggests a different invasion pathway, likely related to maritime trade. Montevideo, a major port city and the capital of Uruguay, serves as an important entry point for global trade. The infestation pattern observed in this city is strikingly similar to the initial stages reported in the city of Buenos Aires, characterised by low attack densities (< 30 entry holes per tree) and concentrations at lower altitudes (< 4 m). Based on the infestation levels observed in sentinel species such as *A. negundo*, the pest may have arrived between the years 2021 and 2022.

The spatial distribution pattern of this quarantine pest within South America shows an extensive distribution spanning approximately 3,500 km of coastline and encompassing diverse climatic zones, due to at least three distinct invasive propagules that may have established independent transmission clusters throughout the Southern Cone. The temporal pattern of these introductions suggests initial establishment in Brazil within the last decade, followed by more recent colonisation events in Argentina and Uruguay, both within the last five years. The detection pattern particularly emphasises the relevance of urban forests *sensu latu* as critical surveillance points for monitoring invasive forest pests. These urban landscapes have effectively served as sentinel networks across the region, enabling early detection and tracking of the pest's spread. The considerable distance between infested localities and major ports of entry suggests that secondary dispersal mechanisms, potentially including human-mediated transport, may play a crucial role in the pest's regional expansion. Management experience from other invaded regions suggests a narrow window of opportunity for effective response and eradication, as it becomes increasingly complex over time [see Paap et al. (2018) and Cook and Broughton (2023)].

Our molecular analyses revealed the presence of a single species of the *E. fornicatus* complex, with COI data aligning with those reported by Ceriani-Nakamurakare et al. (2023) and Covre et al. (2024). Phylogenetic characterisation of the South American populations suggests the presence of a distinct haplotype in this region, with limited genetic similarity (92.2% identity) to invasive populations in other subtropical regions, including South Africa, California and Israel. Instead, the South American specimens demonstrate remarkable genetic affinity (99.4–99.8% identity) with populations from China (Hainan) and recent greenhouse introductions in Europe (Netherlands and Germany). This genetic distinctiveness, coupled with the observed geographic expansion within South America, suggests a unique invasion history and potentially different ecological dynamics compared to other invaded regions. In addition, the existence of novel hosts, pose the question on short- and long-term impacts in the region.

The reported expansion of host plant utilisation by *E. fornicatus* in South America demonstrates its remarkable adaptability and potential impact on regional ecosystems. Our survey revealed fourteen novel host associations across five previously unreported genera, with confirmed breeding activity in nine of these species, in addition to the 602 hosts previously reported at an international level (EPPO 2025). This substantial increase in host species, including the confirmation of breeding in previously reported hosts (*C. speciosa* and *F. excelsior*), suggests a concerning pattern of host range expansion. The surveyed trees were located within the Carlos

Thays Botanical Garden, Buenos Aires, an urban setting where plants are subject to natural environmental conditions. While a formal assessment of plant health (e.g. physiological stress indicators) was not conducted, infestations were observed in both apparently healthy and visibly stressed trees, suggesting that *E. fornicatus* is not strictly limited to weakened hosts in this environment. The pest's remarkable host breadth is particularly noteworthy, given that the identified hosts represent a diverse array of plant families, including both native and exotic species to the region. The fact that native species have been confirmed to be reproductive hosts adds scale to the problem and creates a trade-off for regional pest management. Native species reported as reproductive in the present work extend their distribution beyond human-modified landscapes into natural ecosystems and across neighbouring countries, shaping natural corridors for pest expansion. In addition, native species are an integral part of natural protected areas in regions that are difficult to access and where significant sanitary interventions may not be feasible.

The economic and cultural implications of these findings are amplified by the utilitarian profile of the novel attacked plant species, with eleven species having consumable uses and five serving ornamental purposes, combined with their various symbolic, material and ecological functions and the pest's capability to colonise threatened species (e.g. *F. aspera* and *F. excelsior*, both near threatened as IUCN criteria). The economic consequences of *E. fornicatus* establishment in South America could be substantial, based on experiences from other invaded regions. In Australia, for instance, an eradication scenario involving the removal of infected trees has necessitated an estimated economic investment of approximately ten million US dollars annually for a period of at least three consecutive years (Cook and Broughton 2023). While there have been no reported cases of mortality in affected trees in Montevideo or Rivera, the structural damage caused by gallery excavation and the potential impact of its symbiotic fungi pose significant risks to urban forest health and management costs. This is particularly evident in the urban environments of Montevideo, where it is successfully established on *A. negundo*, *C. cunninghamiana* and *P.* × *acerifolia*. The vertical spread of infestations, as seen in Buenos Aires, Argentina, particularly in *P.* × *acerifolia* up to 16 m height, represents additional challenges for surveillance and control in urban settings, where trees are integrated within complex infrastructure networks.

The significant phytosanitary implications for economically pivotal tree species, including Poplar, Pecan and Avocado, amongst numerous others, underscore the need for a coordinated regional ecological management strategy. The geographical distribution of *E. fornicatus* across the Southern Cone epidemiological landscape underscores the following fundamental needs to be addressed: i) the implementation of robust early detection mechanisms through systematic urban forest and botanical garden surveillance; ii) the standardisation of regional monitoring protocols to ensure comprehensive and comparable data collection; iii) the development of targeted preventative interventions in identified high-risk ecological zones; (iv) the establishment if dynamic, multilateral communication infrastructures between affected nations; and (v) the strategical allocation of research resources to comprehensively evaluate local ecological impacts and develop adaptive control strategies. Currently, this invasive species remains critically under-recognised within South American phytosanitary frameworks and public consciousness, despite its potential to function as a significant ecosystem driver capable of substantially modifying both native and non-native biodiversity dynamics.

Conclusion

The invasion of *E. fornicatus* in South America highlights its rapid spread, host adaptability and unknown introduction pathways, posing significant risks to forestry and biodiversity. The molecular data from Uruguay reveal high genetic similarity to samples from Argentina, Brazil, China (Hainan) and greenhouses in Europe (Netherlands and Germany). The pest's expanding host range, now including fourteen previously undocumented species, emphasises its ecological flexibility and the vulnerability of urban forests and ports as invasion hubs. Shared borders, like in the Rivera-Santana do Livramento complex, may facilitate pest movement despite phytosanitary measures. Coordinated regional actions, including standardised monitoring, early detection and collaborative management, are urgently required to mitigate its growing ecological and economic impacts.

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Author contributions

Esteban Ceriani-Nakamurakare (ECN), Demian F. Gomez (DFG), Ana Trebino (AT), Andrea Listre (AL), Luciana Ingaramo (LI), Agustina Armand Pilón (AAP) and Martin Bollazzi (MB) contributed significantly to the development of this work. Conceptual and methodological work was led by ECN, DFG, AT, AAP and MB. Data curation, formal analysis, investigation and visualisation were primarily performed by ECN, AT, AAP and MB. Funding acquisition was led by ECN and MB, whereas resources were provided by ECN, LI, AL and MB. The original draft was prepared by ECN, DFG and MB, with all the authors contributing to the review and editing process.

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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

Host plant species attacked by *Euwallacea fornicatus* in Argentina, Brazil and Uruguay, including their breeding status, socioeconomic relevance, and IUCN conservation status

Authors: Esteban Ceriani-Nakamurakare, Demian F. Gomez, Ana Trebino, Andrea Listre, Luciana Ingaramo, Agustina Armand Pilón, Martin Bollazzi

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Short Communication

Let ants find them: Using ants as eDNA samplers for detecting the invasive spotted lanternfly

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Abstract

Environmental DNA (eDNA) has emerged as a valuable tool for detecting invasive species, yet its application in terrestrial ecosystems remains challenging due to uneven eDNA distribution. Ants, which forage and consume carbohydrate-rich honeydew from sap-feeding insects, may serve as effective "biological samplers" for invasive species detection. In this study, we evaluated whether ants could facilitate eDNA-based detection of the invasive spotted lanternfly (*Lycorma delicatula*, SLF), given this invasive species is well known for excreting honeydew containing detectable DNA. Worker ants were collected from SLF-infested and non-infested sites and analysed using endpoint PCR and quantitative PCR (qPCR, TaqMan assay) to detect SLF DNA. Both assays successfully detected SLF DNA in 60–100% of ant samples from infested sites, while no SLF DNA was found in ants from non-infested locations. Compared to non-ant insects, ants exhibited higher SLF DNA concentrations, suggesting that honeydew ingestion serves as the primary eDNA source. These findings demonstrate that ants can function as efficient SLF eDNA samplers, providing a scalable and cost-effective alternative to existing SLF detection methods.

Key words: Early detection, eDNA sampler, environmental DNA, foraging behaviour, honeydew, spotted lanternfly

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Introduction

Detecting invasive species using environmental DNA (eDNA) has been shown to enhance detection probability, a crucial factor for improving management efficiency and increasing the likelihood of successful eradication (Mehta et al. 2007; Keller et al. 2022). Unlike aquatic systems, eDNA in terrestrial environments is distributed unevenly. Therefore, identifying efficient methods to target areas where eDNA aggregates is essential for maximising detection success (Eichmiller et al. 2014). A notable example is the detection of the invasive spotted lanternfly (*Lycorma delicatula*, SLF), a highly destructive invasive species in the United States that poses significant agricultural threats (Urban 2020). Leveraging the presence of SLF DNA in its honeydew, Valentin et al. (2020) developed two eDNA collection techniques including spray aggregation (rinsing eDNA from shrubs and understorey vegetation) and tree rolling (removing eDNA from tree trunks and branches using a paint roller). These methods were able to collect SLF DNA from above-ground surfaces and outperformed some traditional SLF detection methods.

Ants are well known for foraging carbohydrate-rich resources, including honeydew from sap-sucking insects (Nelson and Mooney 2022). Their ecological dominance (Parr 2008) and extensive foraging ranges (Adler and Gordon 2003) suggest ants could act as SLF honeydew samplers and even potential amplifiers. Ants can retain liquid food in their gut for extended periods for later sharing with nestmates (Greenwald et al. 2018), further increasing their potential as eDNA reservoirs. If ants can serve as effective SLF eDNA samplers (Fig. 1), this “ant approach” could offer significant advantages over existing SLF eDNA methods by reducing time, device and labour requirements. For example, unlike the two previous eDNA methods requiring specialised equipment and preservation steps, ant specimens can be collected easily and processed directly. This study thus aimed to evaluate whether ants could serve as reliable SLF eDNA samplers. To test this, we analysed ant specimens collected from areas with or without SLF infestations for the presence of SLF DNA using both endpoint PCR and quantitative PCR (qPCR).

Materials and methods

Sample collection and preparation

Samples were collected from a total of six locations in Virginia, including four SLF-infested sites (Lynchburg 1, Lynchburg 2, Harrisonburg and Salem) and two sites with no recorded SLF presence (Blacksburg and South Hill). At each site, five worker ant samples were collected around an infested tree: one sample directly from the tree (e.g. ants foraging on a tree branch) and four additional samples, each from a different direction, at a distance of 5 m from the tree. Aspirators were used to collect all visible ants within a 5-minute period. To compare SLF DNA detection patterns across insect groups, non-ant insects with varying feeding habits were also collected. This comparison allowed us to assess how feeding behaviour influences SLF DNA detection and to determine whether SLF DNA detected in ants resulted from surface contamination (e.g. honeydew adhering to the surface of ants or other insects in an SLF-infested area). The inclusion of non-ant insect samples also helped validate the specificity of the molecular assays, as honeydew from other sap-sucking insects might have been present in the collected ant samples. All insect samples were preserved in absolute ethanol immediately after collection. DNA was extracted from pooled ant specimens (at least three individuals were pooled for extraction) and non-ant insect species (either single insect or pooled specimens consisting of 2–3 individuals) using the E.Z.N.A. Tissue DNA Kit, following the manufacturer’s instructions. To eliminate potential external DNA contamination, all specimens underwent surface decontamination using the bleaching method described by Huszarik et al. (2023).

Molecular assays

Extracted DNA was used as a template for endpoint PCR. We followed the PCR protocol outlined in Kim et al. (2013), targeting the NADH dehydrogenase subunit 6 (ND6) gene of SLF. To ensure amplification specificity, the annealing temperature was adjusted to 56 °C. PCR products were analysed via gel electrophoresis and subsequently verified through Sanger sequencing. The resulting sequences (393 bp) were analysed using BLAST, showing > 99% similarity to published SLF sequences in GenBank, thereby validating the specificity of the primers.

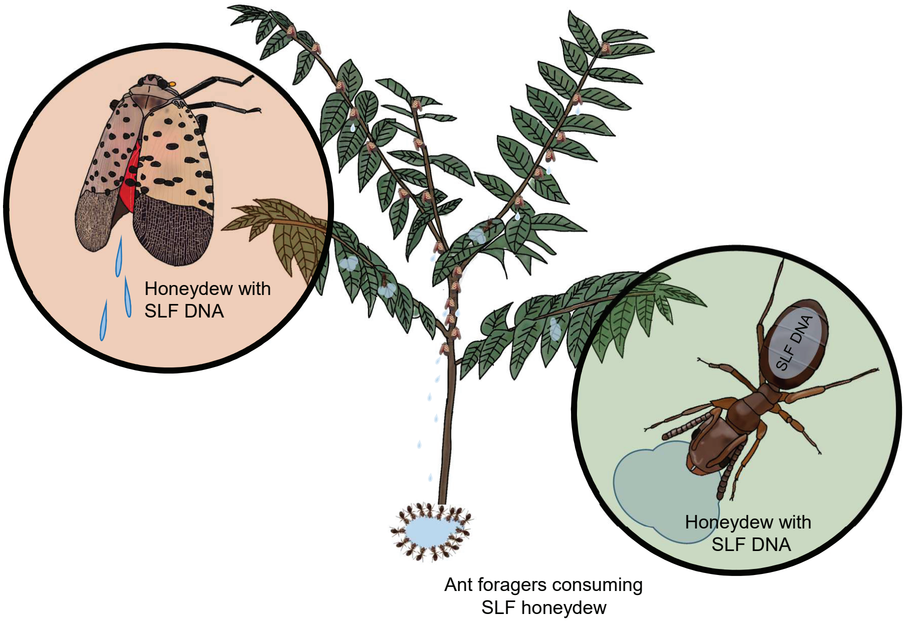

Figure 1. Ant foragers actively feed on and retain honeydew produced by spotted lanternflies (SLF), which may serve as an eDNA source for detection.

For quantitative detection, we employed a TaqMan assay following the protocols and cycling conditions described in Valentin et al. (2020). SLF DNA was analysed in technical triplicates for each sample, alongside a control sample (DNA extracted from an SLF foreleg). The control DNA was quantified using a Qubit™ Flex Fluorometer (Invitrogen, Inc.) and used to prepare a seven-point, 10-fold serial dilution (starting at 14.5 ng/μl) for standard curve generation. SLF DNA concentrations per reaction were estimated, based on the standard curves (efficiencies = 80.35–87.36%; R^2 = 0.9948–0.9952). To ensure the reliability of results, each qPCR run included no-template controls (NTCs) using molecular-grade water as a negative control. One NTC exhibited a Ct value slightly above 43. Samples were classified as positive if they produced a visible band in endpoint PCR or a detectable Ct value (< 41) in qPCR across all triplicates.

Results

Both endpoint PCR and qPCR successfully detected SLF DNA in nearly all the ant samples. Worker ant samples collected from the infested areas showed detection rates ranging from 60–100% using PCR and 80–100% with qPCR (Fig. 2). None of the ant samples collected at the sites with no SLF was positive for SLF DNA using either method (Fig. 2). No SLF DNA was detected in non-ant insect samples (including leafhoppers, lady beetles, stinkbugs and whiteflies) using PCR, consistent with the primer's specificity to SLF. Compered to PCR, qPCR appears to produce higher detection rates in three non-ant insects: leafhoppers (66.6%, 2/3), ladybeetles (50%, 3/6) and stinkbugs (100%, 3/3). However, SLF DNA concentrations detected in ants were higher than in all non-ant insect samples (Fig. 3). While these results may be indicative of the presence of SLF DNA in the non-ant insects, it is important to note that some of these signals may have resulted from short molecular artefacts such as primer dimers.

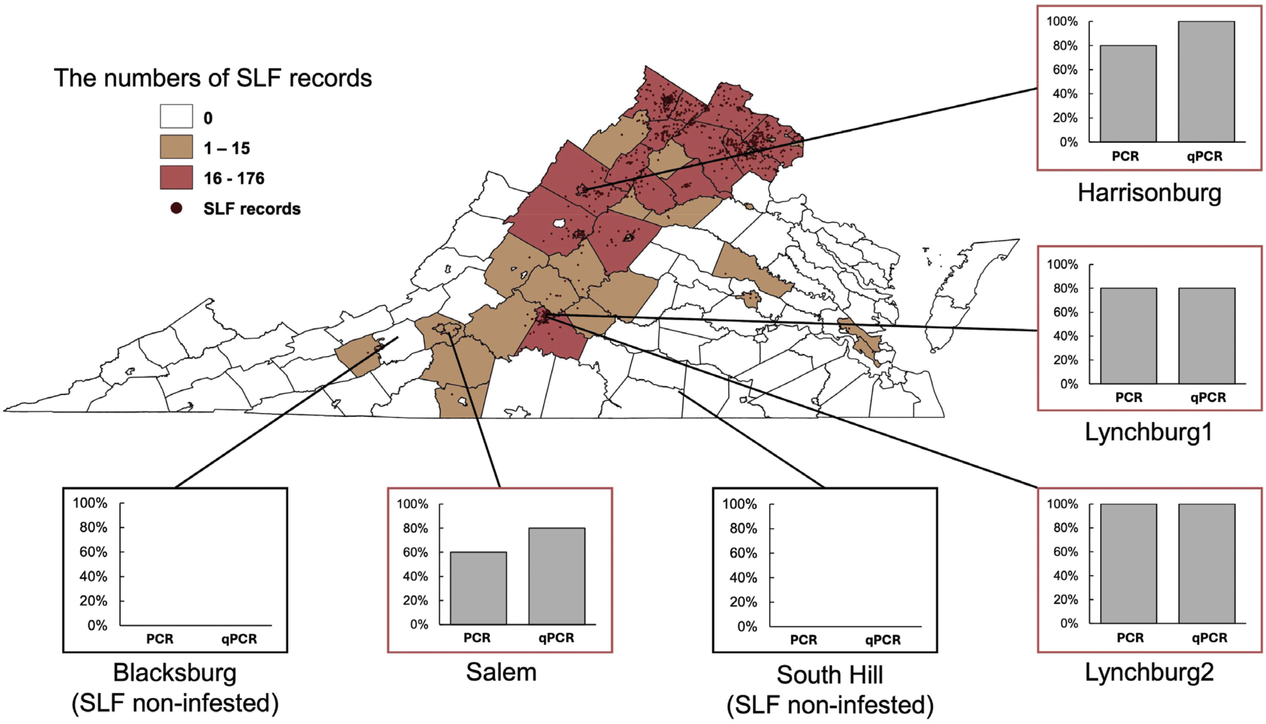

Figure 2. SLF DNA detection rates in ant samples collected from each site, determined by PCR and qPCR (TaqMan assay). A choropleth map of spotted lanternfly (SLF) distribution across Virginia is overlaid with a point density map showing SLF infestation sites. The choropleth layer was generated using QGIS 3.26.3 with kernel density estimation (KDE) and a kernel distance of 20 km. Occurrence data consist of 1,487 human observation records from July 2019 to August 2024 in Virginia, United States (Occurrence download: https://doi.org/10.15468/dl.vurasf, accessed via GBIF.org on 2024-09-12).

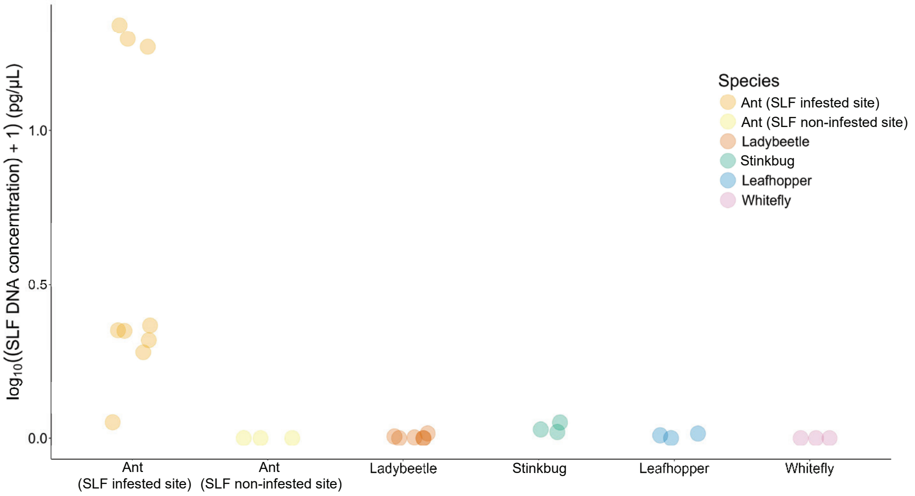

Figure 3. Detection and quantification of SLF DNA in various insect taxa. The detection rates of SLF DNA in ants (including those collected from SLF-infested and non-infested sites), ladybeetles, stinkbugs, leafhoppers and whiteflies were analysed using TaqMan assay.

Discussion

This study demonstrates that ants serve as effective SLF eDNA samplers, as evidenced by their consistently high SLF detection rates using both PCR and qPCR. Compared to non-ant insect species, which generally lack direct interactions with SLF honeydew, ants exhibited significantly higher SLF DNA concentrations. This strongly suggests that honeydew consumption plays a key role in the successful detection of SLF DNA. The effectiveness of ants as SLF eDNA samplers is likely due to their foraging behaviour, which involves actively seeking and retaining carbohydrate-rich foods, including honeydew. This behaviour enables ants to aggregate both honeydew and the DNA contained within it. While we cannot fully rule out the possibility of some SLF DNA detections originating from scavenged SLF tissues, it is unlikely to be the primary source. Worker ants typically transport solid prey back to the nest for larvae to digest, whereas liquid food such as honeydew is retained and shared amongst workers (Greenwald et al. 2018; Fujioka et al. 2023), making the eDNA source in sampled ants more plausible. Leveraging ants' foraging behaviour is particularly advantageous for detecting low-density SLF populations. Early SLF infestations are often cryptic, requiring a method capable of effectively aggregating and concentrating eDNA from the environment. Given ants' extensive foraging ranges (Paulson and Akre 1991), they can cover large areas while searching for food, increasing the likelihood of detecting SLF even when densities are low. While foraging ranges vary across species, some of the ant species (e.g. *Camponotus* ants) collected in this study are known to forage 10–30 m from their nests (Buczkowski 2011). Additionally, their polydomous nature (i.e. colonies are spread across multiple spatially separated nests; Robinson (2014)) may further enhance the detection coverage. This suggests that using ants to detect SLF may extend the detection range beyond the core infestation. Compared to previous eDNA methods (Valentin et al. 2020), the ant approach offers several key advantages: (1) The requirement of filtration or specialised preservation is eliminated, reducing both time and cost; (2) The scalability of this method makes it an ideal candidate for large-scale SLF surveillance. While ants were collected manually using aspirators in this study, well-established ant collection techniques, such as lure stations deployed along transects, could be readily implemented in areas requiring large-scale monitoring; (3) This approach is adaptable across a broad range of environments as ants are widely distributed in diverse habitats. Future research should focus on testing the sensitivity of this method for detecting SLF at low densities and optimising lure-based ant collection techniques to support large-scale monitoring. These efforts will be essential for addressing the ongoing rapid expansion of SLF across the United States.

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Additional information

Conflict of interest

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Ethical statement

No ethical statement was reported.

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Author contributions

W.-J. Lin and C.-C. S. Yang conceived the study; C.-C. S. Yang secured funding and oversaw the project; W.-J. Lin, F.-L. C. Liu, L. Cho and C.-C. S. Yang collected the data; W.-J. Lin and C.-C. S. Yang analysed the data and wrote the manuscript. All authors edited, reviewed and approved the final manuscript for submission.

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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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Research Article

Dunes under attack: untangling the effects of landscape changes on Iceplant invasion (*Carpobrotus* spp., Aizoaceae) in Mediterranean coasts

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Abstract

Invasive alien plants (IAPs) are a great challenge for biodiversity conservation and management. Temporal landscape analysis has a great potential for describing plant invasion process; however, conservation solutions accounting of landscape dynamics are still limited. This research aims to explore the spatial-temporal pattern of *Carpobrotus* spp. by analysing the IAP expansion and reduction processes in relation with landscape changes on Mediterranean coastal dunes. Based on detailed *Carpobrotus* spp. and local land-cover maps of the years 2011 (T0) and 2019–20 (T1), we described coastal dune landscape changes on invaded areas using transition matrices and identified areas of IAP expansion and reduction. We then calculated a set of class and landscape pattern metrics and explored the spatial configuration of invaded patches through trajectory analysis. We also analysed the relationship between *Carpobrotus* spp. patches and landscape pattern over time examining their respective temporal delta values, by Random Forest (RF) models followed by Partial Dependence analysis. The spatial-temporal characteristics of invaded patches and their contextual landscapes varied across coastal tracts experiencing IAP expansion or reduction. Trajectory analysis for IAP expansion areas evidenced an increased *Carpobrotus* spp. cover, accompanied by a rise in patch size, number and connectivity. According to RF models, these trends are related to a morphodynamical stable seashore and increased artificial surfaces. In contrast, trajectory analysis of IAP reduction area evidenced a decline in *Carpobrotus* spp. cover, with patches shrinking into smaller, more regularly-shaped forms. RF models suggest that this reduction is linked to coastal erosion, which compresses dunes against static infrastructures present in the foredune (e.g. roads, building etc.). Temporal landscape analysis provides a sound framework for understanding invasion dynamics across coastal mosaics shaped by the combined effects of factors, such as seashore dynamics and urban sprawl. This approach offers valuable insights for developing tailored management strategies that account for specific contextual nuances and enable informed planning of recovery actions.

Key words: Coastal dune vegetation, coastal erosion and accretion, Invasive Alien Plants, invasion process, landscape change, spatial pattern metrics

Introduction

Biological invasions are a major biodiversity threat (Early et al. 2016; Stoett et al. 2019; Pyšek et al. 2020; Roy et al. 2023) and monitoring and managing them pose significant challenges (Early et al. 2016) partially addressed by global conventions (the Convention on Biological Diversity and the Kunming-Montreal Global Biodiversity Framework, COP 15) and regional legislation (e.g. Regulation EU no. 1143/2014 on Invasive Alien Species). According to the current knowledge and the legislation in force, preventing invasions, for instance of Invasive Alien Plants (IAPs hereafter), needs of rapid, efficient and replicable monitoring approaches to be implemented at different scales (Branquart et al. 2016). Within this framework, the monitoring of both invaded and non-invaded landscapes is crucial for the identification of areas in need of eradication and recovery activities as for early detection and for defining preventative measures (Branquart et al. 2016; Lozano et al. 2023, 2024).

Landscape analysis of invasion processes is important for understanding the spatiotemporal dynamics of each IAP and for defining adequate management strategies (Vaz et al. 2018; Liebhold et al. 2020). In this context, changes in invasion process, whether through expansion or reduction, are strongly influenced by land-use legacies (Malavasi et al. 2014; González-Moreno et al. 2017). For instance, knowing the temporal changes in the abundance and spatial arrangement of habitats more susceptible to biological invasion (Carranza et al. 2011), the land-use categories that facilitate or hinder the dispersal of IAPs propagules (e.g. artificial areas; Basnou et al. (2015); Rodewald and Arcese (2016)) and the elements that enhance or reduce connectivity between suitable areas for IAPs (Glen et al. 2013; Perry et al. 2016) is essential for gaining a deeper understanding of biological invasions across specific landscapes and regions over time (O'Reilly-Nugent et al. 2016). Furthermore, analysing the temporal dynamics of landscape changes is essential for understanding the complex relationships between invasion processes (whether in expansion or reduction) and native vegetation, as well as anthropogenic pressures. Such understanding is essential for developing effective conservation strategies and ecosystem management plans. Additionally, it may aid in identifying priority areas for intervention to prevent and manage IAPs (Branquart et al. 2016).

Amongst the most vulnerable landscapes to biological invasions, coastal dunes are of particular concern both globally (Chytrý et al. 2009) and in the Mediterranean Basin (Cao Pinna et al. 2021). Coastal dune landscapes can be found along approximately three-quarters of the world's shorelines. As complex, transitional and dynamic mosaics, they represent hotspots of highly specialised biodiversity (Martínez and Psuty 2008; Drius et al. 2016). In the Mediterranean Basin, coastal dune landscapes are deeply shaped and fragmented by natural processes amplified by human activities (e.g. coastal erosion, sea level rise; Bazzichetto et al. (2020)) and by human pressure (e.g. urbanisation, tourism etc.; Doody (2004, 2013); Malavasi et al. (2013)) both "squeezing" dune zonation to simplified small relict areas.

Amongst the worst IAPs impinging Mediterranean coastal landscapes, the *Carpobrotus* species (Aizoaceae) are of particular concern (Campoy et al. 2018). *Carpobrotus* spp. invasion on coastal dunes, alters plant diversity, negatively affecting the germination, survival, growth and reproduction of native species (Mugnai et al. 2022) and transforms key soil physical and chemical properties including soil PH, salt content, moisture levels, nutrient content and microbial activity (Rodríguez-Caballero et al. 2020). *Carpobrotus* spp. invasion on coastal landscapes is shaped by the interplay of biotic factors (e.g. competition with native species, dispersal by native and introduced animals), abiotic features (e.g. coastal erosion and accumulation, seashore distance) and anthropogenic pressure (e.g. dune trampling, land take, artificial infrastructures; Bazzichetto et al. (2018a, b)). Consequently, factors such as seashore erosion and accretion (as discussed by Bazzichetto et al. (2020)) and different urban processes, such as expansion or stability (as outlined by Malavasi et al. (2018a)), may drive landscape dynamics and, in turn, influence invasion processes in contrasting ways. Given the extensive presence of *Carpobrotus* spp. on the Mediterranean coasts and their significant threat to biodiversity, considerable efforts have been directed towards intensive monitoring, detection and mapping (Underwood et al. 2003; Innangi et al. 2023), as well as for analysing their spatial pattern (e.g. Carranza et al. (2010); Malavasi et al. (2014)).

Monitoring and mapping of the *Carpobrotus* spp. distribution is crucial for developing and implementing targeted management and invasion control strategies (Lazzaro et al. 2020, 2023).

However, monitoring IAPs on dynamic dune mosaics by traditional approaches performed through field campaigns is both resource-intensive and costly. The field campaigns often cover limited areas due to accessibility constraints and historical distribution maps required for monitoring invasion dynamics at landscape scale are frequently unavailable (Müllerova et al. 2017; Cascone et al. 2021; Charbonneau et al. 2023). Consequently, the temporal changes of invasion process were barely explored and focused on limited number of IAP as *Acacia saligna* (Kutiel et al. 2004), *Carex arenaria* (Nielsen et al. 2011), *Oenothera drummondii* (Gallego-Fernández et al. 2019), *Carex kobomugi* (Charbonneau et al. 2020).

On the contrary, the increasing availability of remote sensing data is fundamentally transforming the monitoring of alien invasion processes on landscapes, offering the promise of advanced tools for tracking invasion dynamics across various spatial and temporal scales (Villalobos Perna et al. 2023). Such remote sensing tools may aid in dealing with new issues as the analysis of the intricate relationship between the temporal evolution of invasion patterns and the changes in landscape composition and configuration within complex environments such as coastal dunes.

In consideration of the above, the present research aims to analyse the spatial-temporal changes of *Carpobrotus* spp. invasion by analysing the invasion process of expansion and of reduction in relation to the landscape context pattern (i.e. composition and configuration) which occurred during one decade (2011 – 2019–20) in Mediterranean coastal dunes. Based on detailed bi-temporal land-cover maps and transition matrices analysis, we have described changes in the coastal dune mosaic and have addressed two main questions: (i) How does the *Carpobrotus spp.* invasion pattern vary within the coastal dune landscapes shaped by different factors (e.g. coastal erosion, urban expansion and ecosystems fragmentation)? (ii) How do the temporal changes of *Carpobrotus* spp. invasion pattern relate to changes in the composition and configuration of other cover classes within coastal dune landscapes?

We have hypothesised that the IAPs spread, establishment and growth are not uniform, but vary across landscapes shaped by different environmental variables (e.g. seashore dynamics, urban sprawl).

Materials and methods

Study area

The study area encompasses a representative coastal landscape of Mediterranean Holocene dunes on the Tyrrhenian coast in Central Italy (Lazio Region) of approximately 280 km (Fig. 1a; Mazzini et al. 1999; Amato et al. 2012). These dunes that are low and narrow and occupy a 400–500 m wide strip parallel to the shoreline (Acosta et al. 2003), in natural conditions, host a well-developed vegetation zonation, which follows a steep sea-inland abiotic gradient from pioneer communities dominated by annual plants to the inner sectors of Mediterranean scrub (Acosta et al. 2003; Fig. 1b). In addition to alien plant invasions (Bazzichetto et al. 2018a), the analysed dunes are highly threatened by coastal erosion (Bazzichetto et al. 2020), urban expansion (e.g. new buildings, recreational sites etc.), land take (e.g. agricultural expansion, afforestation, industrial and harbour development etc.) and ecosystems fragmentation (Malavasi et al. 2014, 2018a).

Carpobrotus acinaciformis (L.) L.Bolus and *C. edulis* (L.) N.E.Br. are mat-forming trailing succulent perennial herbs that are native to South Africa (Wisura and Glen 1993). Since both species tend to invade similar coastal dune habitats with comparable behaviour and impacts (Sarmati et al. 2019) and they commonly hybridise with each other, in their invasive range, they are frequently referred to as

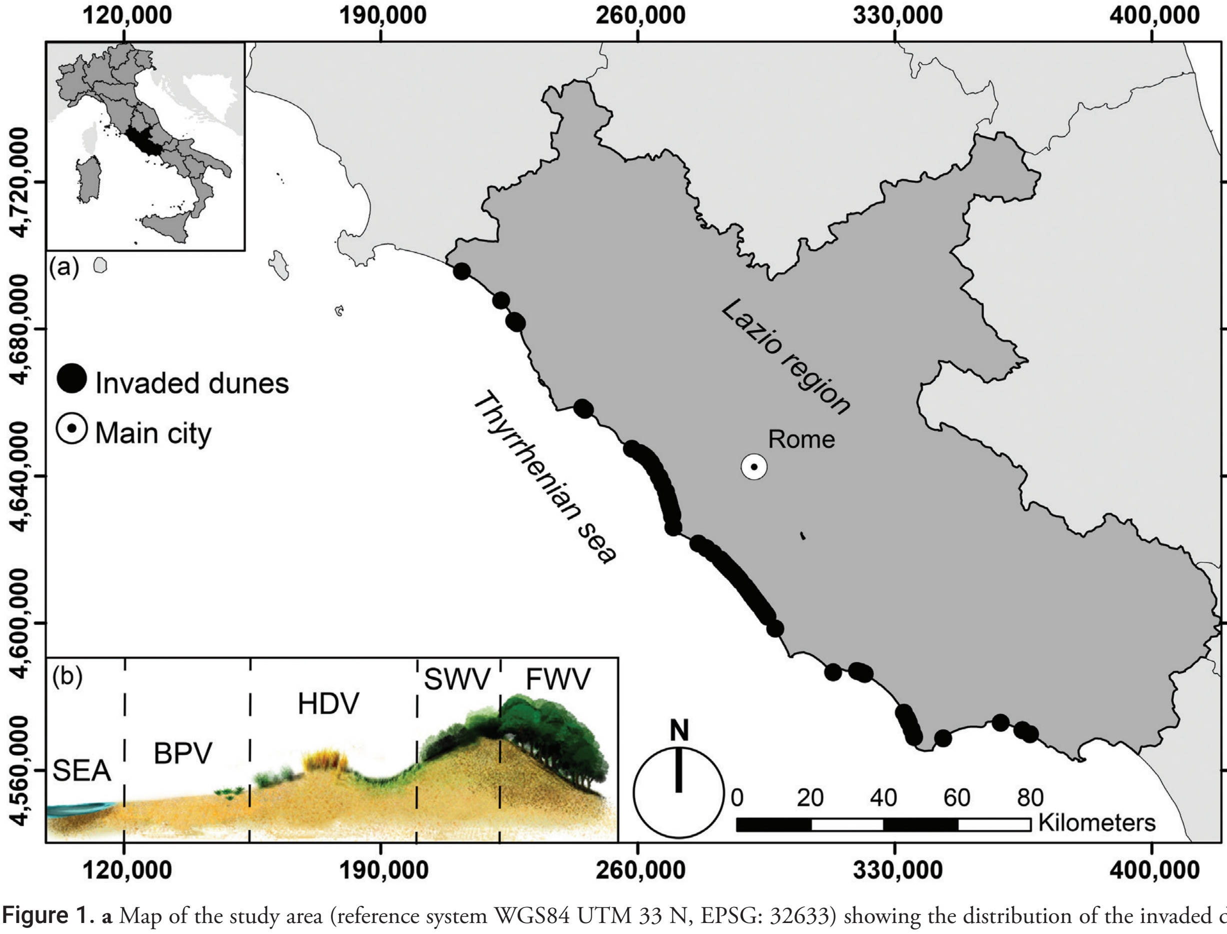


Figure 1. **a** Map of the study area (reference system WGS84 UTM 33 N, EPSG: 32633) showing the distribution of the invaded dune systems **b** schematic profile of coastal dune zonation with the acronym of the mapped natural coastal classes: SEA – sea water, BPV – beach with pioneer vegetation, HDV – herbaceous dune vegetation, SWV – Shrub woody vegetation, FWV – forest woody vegetation.

Carpobrotus spp. (Novoa et al. 2023). In the study area, *Carpobrotus acinaciformis* (L.) L.Bolus, *C. edulis* (L.) N.E.Br. and probably also hybrids between the two, were introduced as ornamental plants and for consolidating dunes (Campoy et al. 2018). As on other sectors of their invasive range, *Carpobrotus* spp. tend to invade herbaceous dune vegetation growing on shifting and fixed dunes preferentially and, to a lesser extent, clearings in shrubland and understoreys in woody vegetation and fore dunes (Carranza et al. 2011; Bazzichetto et al. 2018b).

Data collection and analysis

The workflow for analysing changes in invaded coastal landscapes and for assessing the interplay between invaded patch dynamics and contextual landscapes is illustrated in Fig. 2. It includes four key steps: (A) bi-temporal *Carpobrotus* spp. and land-cover mapping, (B) coastal dune landscape change, (C) spatial pattern analysis over time and (D) relationship between IAP dynamics and coastal landscape pattern.

Bi-temporal *Carpobrotus* spp. and land cover mapping

We selected two free web-mapping services providing spring/summer RGB orthophotos with a spatial resolution below 1 m to visually map *Carpobrotus* spp. patches greater than 1 m^2 across both past and present coastal dunes of Lazio Region (Fig. 2A) using a GIS environment (QGIS 3.22). Specifically, the imagery used (Suppl. material 1: table S1) included: spring and summer aerial RGB orthophotos from 2011 (T0 hereafter) available at the Italian National Geoportal (http://www.pcn.minambiente.it/mattm/servizio-wms/); and spring and summer satellite RGB orthophotos of the years 2019–20 (T1 hereafter) provided by Google Earth (Maxar Tecnhologies/Airbus, Inc.). To minimise the influence of seasonal changes on Mediterranean coastal landscapes (e.g. winter storms, tide etc.), we mapped *Carpobrotus* spp. patches during the same months (May-August) in T0 and in T1. Additionally, we addressed potential co-registration issues between the T0 and of T1 orthophotos by calculating the Root Mean Square Error (RMSE) of coordinate differences for 100 control points located in stable landscape features (e.g. buildings, road crossings, pools etc.; Coulter and Stow (2008)). This analysis revealed a georeferencing difference of 0.43 m, which is below the 0.50 m threshold considered indicative of very fine co-registered orthophotos (Talavera et al. 2022) and, thus, the alignment of the orthophotos used in this study was assumed as acceptable.

We mapped a total of 486 *Carpobrotus* spp. patches in T0 and 497 patches in T1. The *Carpobrotus* spp. patches were grouped into coastal tracts within non-overlapping circular buffer areas (Zuckerberg et al. 2020), each with a radius of 100 m (Fig. 2A). These buffer areas (hereafter tracts) were defined with the same centroid for both dates and include an extent widely used in coastal dune ecosystems where the relationship between landscape composition and biodiversity is evident (Bezzi et al. 2018; Malavasi et al. 2018a; Sperandii et al. 2019). Within each coastal tract containing *Carpobrotus* spp. patches, we generated a fine-scale (1:2000) land-cover/vegetation map (Fig. 2A) at the fourth level of detail of the CORINE land-cover legend (CL, Table 1, Acosta et al. (2005)). The semi-natural and natural cover types correspond to habitat types of European conservation concern (Annex I of the Habitats Directive 92/43/EEC; Table 1; Malavasi et al. (2013)). The accuracy assessment of the land-cover maps was based on 880 random control points (440

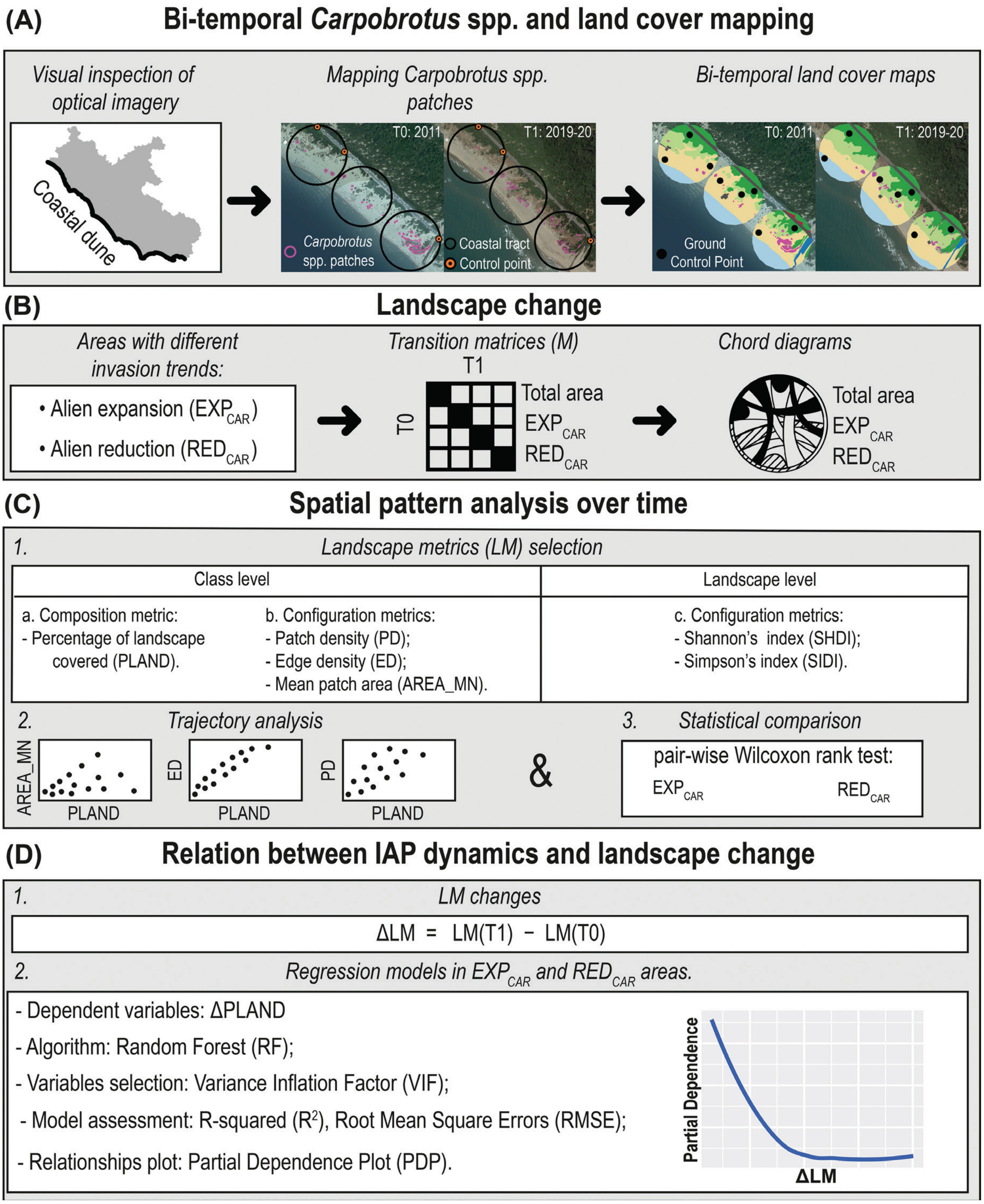


Figure 2. Schematic overview of the procedure implemented to analyse the spatiotemporal changes of *Carpobrotus* spp. invasion in relation with the landscape context. **A** Bi-temporal mapping of *Carpobrotus* spp. and land cover **B** analysis of landscape change **C** temporal spatial pattern analysis **D** assessment of the relationship between IAP dynamics and coastal landscape pattern.

points in T0, 440 points in T1). For the T0 map, the land-cover class for each control point was assigned using an existing detailed land-cover map (Carranza et al. 2008). For the T1 map, the classification of each point was determined by a dedicated field campaign carried out in Spring 2023. To evaluate the accuracy of the maps, confusion matrices were constructed and the following performance metrics were calculated: Overall Accuracy (OA%), Producer Accuracy (PA%), User Accu-

Table 1. Land-cover acronym, along with a detailed description (including Habitats of Conservation concern ex Dir. No. 92/43/EEC), CORINE land-cover category and the relative hierarchical code.

Acronym	Detailed description	CORINE category	CORINE code
ART	ARTificial areas including building, streets, urban fabrics, industrial surfaces.	Artificial areas	1.
BPV	Beach with Pioneer annual Vegetation. (EU 1210: annual vegetation of drift lines) and open sand without vegetation.	Open Sand	3.3.1.1.
HDV	Herbaceous Dune Vegetation growing on fore dunes. (EU 2110: embryonic shifting dunes, EU 2120: shifting dunes along the shoreline with *Ammophila arenaria*, EU 2210: *Crucianellion maritimae* fixed beach dunes, EU 2230: Malcolmietalia dune grasslands).	Partially vegetated dunes and densely vegetated dunes	3.3.1.2.
SDV	Shrub Dune Vegetation growing on fixed dunes. (EU 2250*: fixed coastal dunes with *Juniperus* spp., EU 2260: *Cisto-Lavenduletalia* dune sclerophyllous scrubs).	Mediterranean maquis	3.2.3.1.
FDV	Forests and woody Dune Vegetation. (EU 9340: *Quercus ilex* and *Quercus rotundifolia* forests, EU 2270*:wooded dunes with *Pinus pinea* and/or *Pinus pinaster*).	Forest	3.1.
SHV	Semi-natural Herbaceous Vegetation: abandoned meadows and pastures with different degrees of degradation or recolonisation.	Semi-natural herbaceous and ruderal vegetation	3.2.4.2.
WET	WETland non-forested areas of low-lying land flooded by fresh stagnant or circulating water.	Coastal Wetlands	4.2.
SEA	Tyrrhenian SEA.	Marine waters	5.2.
CAR	Invaded patches of *Carpobrotus acinaciformis, C. edulis* or their hybrids.	–	–

racy (UA%), Cohen's Kappa statistics (K), True Kill Statistic (TSS) and, given the possibility of unbalance data, we calculated also Balanced Accuracy (BA), a reliable metric to assess the performance of classification models on imbalanced datasets (Velez et al. 2007; Congalton and Green 2019).

Coastal dune landscape change

Based on the temporal dynamics of *Carpobrotus* spp. patches, we analysed overall invasion trends, ranging from nearly stable tracts to those exhibiting maximum expansion or reduction (from T0 to T1), capturing the full spectrum of landscape dynamics within the coastal dunes of the Lazio Region (Suppl. material 1: fig. S1). It is important to note that contrasting temporal trends, such as the expansion and reduction of *Carpobrotus* spp., may be driven by different environmental forces that influence landscape processes in distinct ways. Factors like seashore erosion and accretion (Bazzichetto et al. 2020) and varying levels of urban expansion or stability (Malavasi et al. 2018a) can shape landscape dynamics and, consequently, invasion processes, in opposing directions. To enhance our analysis, we categorised coastal tracts into two groups: a) *Carpobrotus* spp. expansion (hereafter $\mathrm{EXP}_{\mathrm{CAR}}$), including 60 tracts where the IAP cover increased and b) *Carpobrotus* spp. reduction (hereafter $\mathrm{RED}_{\mathrm{CAR}}$) gathering 35 tracts where the IAP cover decreased (Fig. 2B). By conducting separate analyses of $\mathrm{EXP}_{\mathrm{CAR}}$ and $\mathrm{RED}_{\mathrm{CAR}}$ tracts, we improved our understanding of the ecological connections between invasion dynamics and the broader landscape context in which invasive alien plants are embedded (Carranza et al. 2010; Malavasi et al. 2014; Bazzichetto et al. 2018b).

Landscape change for $\mathrm{EXP}_{\mathrm{CAR}}$ and $\mathrm{RED}_{\mathrm{CAR}}$ tracts was analysed by transition matrices comparing landscape cover classes in T0 and T1. The stability (transition matrix diagonal), the dynamism (other matrix elements) and the direction of change on $\mathrm{EXP}_{\mathrm{CAR}}$ and $\mathrm{RED}_{\mathrm{CAR}}$ coastal tracts were summarised by Chord diagrams (Fig. 2B; Gu et al. (2014)). As the outer ring of the chord diagram represents the extension of land-cover classes in T0, the internal arrows (e.g. size and direction) indicate the transitions occurred towards other classes in T1. We used the R package "circlize" and the function chordDiagram to create the diagram (Gu et al. 2014).

Spatial pattern analysis over time

Changes in the spatial pattern of the coastal tracts invaded by *Carpobrotus* spp. were assessed by calculating and comparing, over time, a comprehensive set of landscape metrics (LM) that depict spatial composition and configuration at both the landscape level (LM_{land}) and the class level (LM_{class}) for the different time steps (T0 and T1; Fig. 2C; Table 2; Riitters et al. (1995)). We selected metrics able to depict distinct ecological processes within dune landscape patterns, whether these processes impede or facilitate plant invasion. These metrics may exhibit non-linear correlation issues and encompass key ecological processes and mechanisms essential for advancing the ecological understanding of *Carpobrotus* spp. pattern of change (Smith et al. 2009; Long et al. 2010). In particular, the selected metrics capture critical drivers of the invasion process on coastal dunes, including dune fragmentation and urban expansion (Malavasi et al. 2014, 2018a; Carranza et al. 2015), erosion/accretion dynamics (Bazzichetto et al. 2020), the loss of integrity within natural dune mosaics (Acosta et al. 2003) and the presence of both natural and artificial corridors facilitating alien propagule dispersal (Bazzichetto et al. 2018b).

For landscape metrics (LM_{land}), we calculated and compared over time two indices depicting landscape richness (number of land-cover classes) and evenness (relative abundance of each class). As Shannon index (SHDI), including a logarithmic transformation of abundance values (Table 2), is particularly sensitive to less abundant classes and constitutes a good indicator of equipartition, Simpson's index (SIDI; Table 2), is a reliable measure of dominance (McGarigal et al. 2012).

For class metrics (LM_{class}), we calculated and compared over time four indices, illustrating spatial composition (class abundance) and configuration (class spatial pattern, see Table 2; McGarigal et al. (2012)). Amongst these class metrics, PLAND, representing the percentage of the landscape covered by a given class, is a good surrogate for class dominance or rareness. PD, or patch density, describes the number of patches per unit area and measures class aggregation or dispersion in the landscape. ED, or edge density, calculated as the ratio between class perimeter and landscape area, measures class shape complexity or simplicity (e.g. edge effects vs. core areas) and provides an overview of the contact of a class with other land cover classes (e.g. high values may suggest the role as landscape matrix). AREA_MN, measured as the mean patch dimension of a given class, depicts the presence of large or small patches and is a good indicator of natural habitats fragmentation (e.g. smaller patches of native vegetation), as well as of invasive plants colonisation (e.g. increasing size of alien species patches over time; Table 2; McGarigal et al. (2012)). Metrics were calculated with FRAGSTAT 4.2 software (McGarigal et al. 2012).

Spatial pattern changes over time at class level ($LM_{classT0}$ vs. $LM_{classT1}$) were analysed by trajectory analysis (*sensu* Long et al. (2010)) depicting the relationship between composition and configuration metrics (Carranza et al. 2015; Malavasi et al. 2018b). For each class, a specific bi-temporal relationship space was produced by projecting in a Cartesian diagram the class configuration metric values (PD, ED, AREA_MN) computed for each coastal tract against the respective percentage of class cover (PLAND). Then, the arithmetic mean of each class metric

Table 2. Names (acronyms), formulas, descriptions, units of measurement and the associated spatial pattern levels (Class/Landscape) and facets (composition/configuration) of the selected pattern metrics. A = total landscape area, nj = number of patches of j-land-cover class, aij = area of the i-th patch of j-land-cover class, eij = total length of the i-th patch edge of j-land-cover class, m = total number of land-cover classes, Pj = proportion of the landscape occupied by j-land-cover class.

Name (Acronym)	Formula	Description	Unit / Range	Pattern facet
Class level (LM_{class})				
Percentage of Landscape (PLAND)	$\frac{\sum_{i=1}^{n_j} a_{ij}}{A} * 100$	Sum of the areas (m^2) of all patches of the *j*-land cover class, divided by coastal tract area (m^2) in percentage. Measure of dominance/rareness.	Percent (%) 0 ≤ PLAND < 100	Composition
Patch density (PD)	$\frac{n_j}{A}$	Density of patches of the *j*-land-cover class per unit area. Measure of aggregation/dispersion.	Number/ha PD > 0, no limit.	Configuration
Edge density (ED)	$\frac{\sum_{i=1}^{n_j} e_{ij}}{A}$	Edge length of *j*-land-cover class on the landscape area. Length of the contact with other classes. Measure of shape complexity/simplicity.	Metress/ha ED > 0, no limit	Configuration
Mean patch area (AREA_MN)	$\frac{\sum_{i=1}^{n_j} a_{ij}}{n_j}$	Area of *j*-land-cover class divided by its number of patches. Measure of fragmentation/colonisation.	Ha AREA_MN > 0, no limit	Configuration
Landscape level (LM_{land})				
Shannon Diversity Index (SHDI)	$-\sum_{i=1}^{m} (P_i{}^{\star} \ln P_i)$	Shannon's Diversity Index accounting for land-cover class richness and equitability. Sensitive to rare land-cover classes.	Natural number 0 ≤ SHDI < ∞ SHDI = 0 – no diversity	Composition
Simpson Diversity Index (SIDI)	$1-\sum_{j=1}^{m} P_j^2$	Simpson's Diversity Index depicting land-cover class richness and dominance. Sensitive to dominant land-cover classes.	0 ≤ SIDI <1. SIDI = 0 – no diversity	Composition

(mean_LM$_{class}$) was plotted in the relationship space and the temporal trajectories for EXP$_{CAR}$ and RED$_{CAR}$ were drawn by connecting metric means chronologically with arrows. After a visual inspection of the relationship space to ascertain the means can be statistically compared, we assessed pattern landscape and class metric changes (LM$_{classT0}$ vs. LM$_{classT1}$ and LM$_{landT0}$ vs. LM$_{landT1}$) by the non-parametric pairwise Wilcoxon rank test.

The temporal changes in composition given by the mean values of PLAND (e.g. in PLAND$_{CAR}$, PLAND$_{HDV}$, PLAND$_{ART}$ etc.) and configuration metrics assessed as the mean of PD, ED and AREA_MN (e.g. in PD$_{CAR}$, ED$_{HDV}$, AREA_MN$_{ART}$ etc.) were interpreted accounting of the specific non-linear relationship amongst them (Fig. 3; Long et al. (2010)). Given that numerous previous studies have demonstrated the non-linear correlation between landscape composition (PLAND) and configuration metrics (PD, ED, AREA_MN) across different environments, such as grasslands, forests, croplands and urban areas (Long et al. 2010; Su et al. 2012; Carranza et al. 2015; Malavasi et al. 2018b; Hermosilla et al. 2019; Zhang et al. 2020), it is essential to simultaneously analyse their temporal changes (Fig. 3). Such a concurrent interpretation of pattern metrics facilitates a nuanced understanding of the intricate dynamics interweaving these landscape facets (Long et al. 2010; Carranza et al. 2015).

In general, as described by Long et al. (2010), an increase in patch density (PD) accompanied by a decrease in PLAND values (e.g. numerous smaller patches) may indicate the fragmentation of natural habitats (Fig. 3a, Carranza et al. (2015)). Conversely, a simultaneous rise of PD and PLAND (e.g. numerous, larger patches) may reflect colonisation processes (Fig. 3a). Furthermore, a decrease in

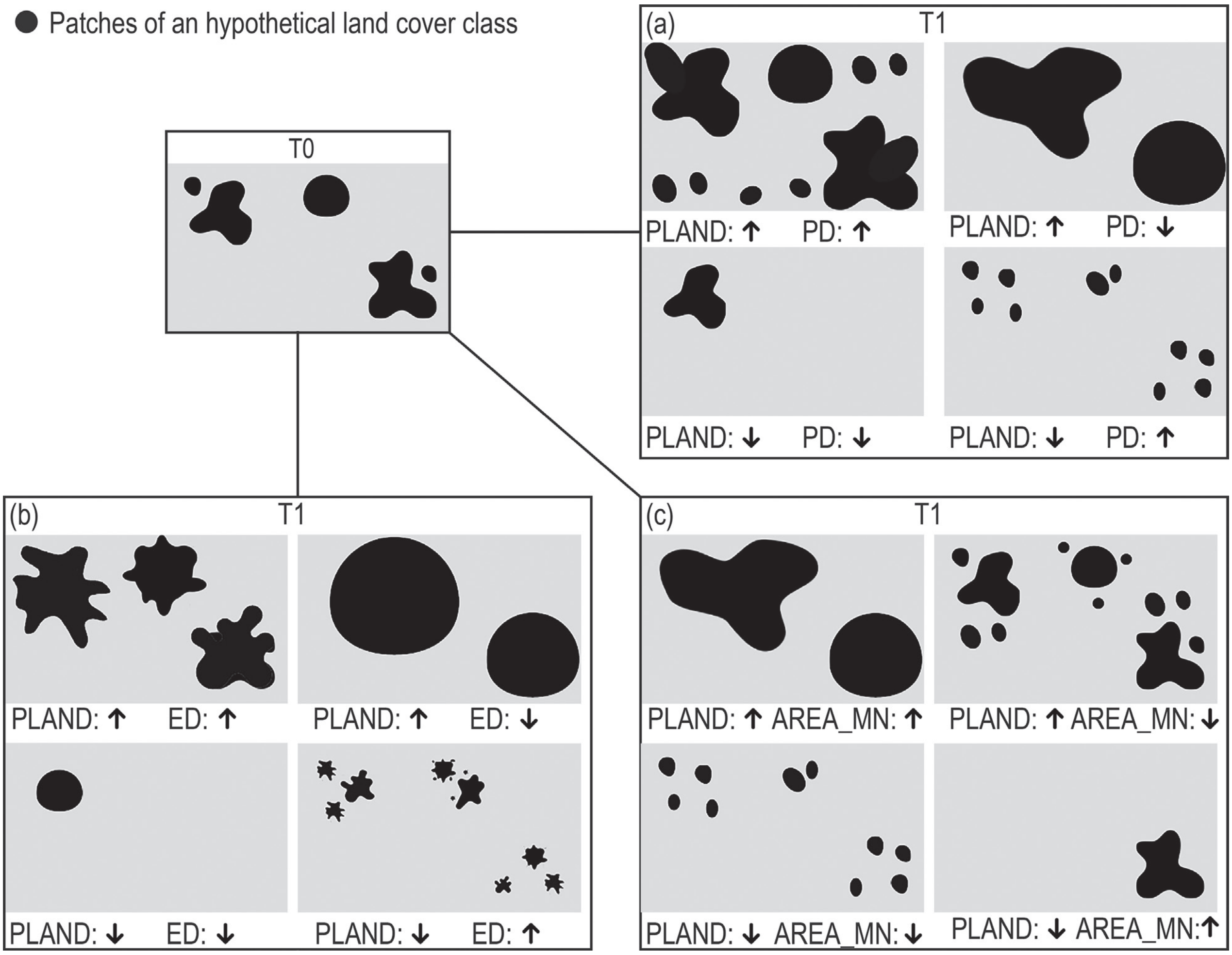


Figure 3. Combined effects of temporal changes (from T0 to T1) in composition (e.g. increasing or decreasing PLAND values) and configuration (e.g. increasing or decreasing PD, ED, AREA_MN values) of a hypothetical land-cover class: **a** changes in PLAND and PD **b** changes in PLAND and ED **c** changes in PLAND and AREA_MN.

PD could signify either increasing fragmentation (if accompanied by a reduction in PLAND) or habitat expansion due to the aggregation of several small patches into fewer, larger ones (if accompanied by PLAND increase; Yang and Mountrakis (2017)). Similarly, a rise in ED values can correspond to two scenarios (Fig. 3b). A simultaneous increase of ED and PLAND (e.g. larger and more irregularly-shaped patches) is likely associated with habitat frontal expansion. On the other hand, an increase in ED combined with a decline in PLAND (e.g. smaller irregularly-shaped patches) may indicate habitat fragmentation caused by patch shrinkage and irregular edge erosion (Carranza et al. 2015). On the other hand, decreased ED values can be associated with fragmentation if accompanied by a reduction in PLAND or with habitat expansion and the aggregation of irregular patches into larger, more regular ones if accompanied by an increase in PLAND (Yang and Mountrakis 2017). With regard to AREA_MN (Fig. 3c), a decline in average patch size combined with increasing PLAND may indicate habitat expansion with the formation of new small patches. Conversely, the simultaneous reduction of AREA_MN and PLAND (e.g. smaller patches) suggests fragmentation of larger patches into smaller ones, along with a reduction in the cover of the remaining patches (Long et al. 2010; Carranza et al. 2015). An increase in AREA_MN and PLAND (e.g. lager patches and higher dominance in the landscape) may indicate habitat expansion.

In contrast, an increase in AREA_MN accompanied by a reduction in PLAND reduction (e.g. large residual patches) may depict habitat loss, characterised by the disappearance of several medium-to-small patches and the persistence of one or few large patches (Malavasi et al. 2018b).

Relationship between coastal dune and IAP dynamics

To analyse the relationship between the spatial-temporal dynamics of invasion and the changes occurring on dune landscape, we computed the delta values of IAP pattern metrics ($\Delta LM_{CAR} = LM_{CAR}$ in T1 – LM_{CAR} in T0) and of coastal mosaic pattern ($\Delta LM = LM_{T1} - LM_{T0}$; Fig. 2D).

The visual inspection of bi-plots reporting changes on *Carpobrotus* spp. metrics (ΔLM_{CAR}) and landscape indexes (e.g. ΔLM_{class} and ΔLM_{land}) evidenced non-linear relations, so we explored invasion dynamics and landscape changes adopting a machine-learning approach organised in the following steps (Suppl. material 1: figs S2–S9). First, we computed the Variance Inflation Factor (VIF) on the delta of all the pattern variables and removed from further analysis those with high multicollinearity (VIF values ≥ 3), which can cause overfitting problems; then, we analysed the relationship of *Carpobrotus* spp. spatial-temporal pattern (ΔLM_{CAR}) with coastal dune landscape change (ΔLM_{class}, ΔLM_{land}) using Random Forest algorithm (RF; Breiman 2001) and we displayed the results using Partial Dependence Plot (PDP; Friedman 2001).

Specifically, we implemented a series of RF models (four for areas experiencing alien expansion: EXP_ΔPLAND$_{CAR}$, EXP_ΔPD$_{CAR}$, EXP_ΔED$_{CAR}$, EXP_ΔAREA_MN$_{CAR}$ and, four in areas undergoing alien reduction: RED_ΔPLAND$_{CAR}$, RED_ΔPD$_{CAR}$, RED_ΔED$_{CAR}$, RED_ΔAREA_MN$_{CAR}$). RF was implemented using the following settings: i) high number of uncorrelated decision trees (*Ntree* = 1000); ii) increasing number of variables randomly selected at each node of the decision tree (*Mtry* ranging from 2 to the total number of variables); iii) minimum number of observations in a terminal node (minimal node size, *MNS*: from 1 to 5; Probst et al. (2018)). Each RF model (using the R package 'caret', function train; Kuhn (2008)) was computed using a 10-fold cross-validation and we selected for further analysis the model with the highest coefficient of determination (R^2). The performance of the RF models was evaluated using the coefficient of determination (R^2) and root mean square error (RMSE; Routh et al. (2018)). The relative importance of pattern metrics' change (ΔLM_{class} and ΔLM_{land}) in the RF models was determined using the Mean Decrease Importance (MDI) index (i.e. the Gini index with the sum of squares as an impurity measure). The marginal effects of landscape dynamics (ΔLMclass and ΔLMland) on *Carpobrotus* spp. spatial pattern changes (ΔLM_{CAR}) in the RF models (holding other variables constant, for example, median; Friedman (2001)) were descried by Partial Dependence Plots (PDP).

Results

Land-cover maps accuracy and transition matrix analysis

The produced land-cover maps are highly accurate (OA, K, TSS and BA greater than 85.682%, 0.833, 0.773 and 0.886, respectively; Suppl. material 1: tables S2, S3) and, thus, reliable for further landscape analysis. A comparison of land-cover maps between T0 and T1, using transition analysis, revealed changes in the 18% of the landscape, with most land-cover classes shifting towards the neighbouring categories in a comparable manner (Fig. 4a–c; Suppl. material 1: table S4).

In both years, the dominant categories are Artificial areas (ART) and Open Sand (BPV) summing up to over the 40% of the mapped area, followed by herbaceous dune vegetation (HDV) and Sea (SEA) covering over 12% (Fig. 4a; Suppl. material 1: table S4). In EXP_{CAR} coastal tracts, the landscape was relatively stable with weak accretion processes (reduction of SEA class area; Fig. 4b), while in RED_{CAR}, landscape tracts have changed with a consistent increase in the SEA category at the expense of BPV (Fig. 4c; Suppl. material 1: tables S5, S6). Four cover classes (SDV, SHV, WER, FDV), which had limited extension in both time steps, were excluded from further analysis and modelling.

The chord diagram of the overall landscape evidences balanced shifts between CAR and HDV (Fig. 4a) differently, the separate analysis of chord diagrams revealing opposite landscape dynamics on EXP_{CAR} and RED_{CAR}. In EXP_{CAR} areas, we observed the expansion of CAR class at the expense of the HDV class (Fig. 4b), while in RED_{CAR} landscapes, we registered HDV replacing *Carpobrotus* spp. class; Fig. 4c).

Spatiotemporal trends of *Carpobrotus* spp.

The spatial pattern of *Carpobrotus* spp. patches changed significantly over time (from T0 to T1) presenting opposite trends in EXP_{CAR} and RED_{CAR} tracts (Fig. 5). In EXP_{CAR} tracts, *Carpobrotus* spp. patches increased in extent ranging from 0.41 m^2 to 865.84 m^2. Conversely in RED_{CAR} tracts, the extent of *Carpobrotus* spp. decreased ranging from – 1.65 m^2 to – 1322.25 m^2 (Suppl. material 1: fig. S10).

In tracts of IAP expansion (EXP_{CAR}), all *Carpobrotus* spp. spatial metrics significantly increased. The extension of invaded areas increased (greater $PLAND_{CAR}$, Fig. 5a–d) with *Carpobrotus* spp patches distributed on larger ($AREA\_MN_{CAR}$, Fig. 5a, b), more numerous (PD_{CAR}, Fig. 5a, d) and irregularly-shaped patches (ED_{CAR}, Fig. 5a, d). On EXP_{CAR} landscape, we also registered a significant increase of artificial patch dimension ($AREA\_MN_{ART}$; Suppl. material 1: fig. S11). On the other hand, in coastal tracts of IAP reduction (RED_{CAR}), the decline of *Carpobrotus* spp. ($PLAND_{CAR}$; Fig. 5a–d) coincides with simpler configuration metrics, indicated by lower values in $AREA\_MN_{CAR}$ (Fig. 5a, b) and ED_{CAR} (Fig. 5a, c).

Spatiotemporal trends of *Carpobrotus* spp. in relation to landscape changes

Changes on *Carpobrotus* spp. pattern (ΔLM_{CAR}) are related with landscape changes (ΔLM_{land}), specifically concerning sea (SEA), herbaceous dune vegetation (HDV), the artificial surfaces (ART) classes (Figs 6, 7, Suppl. material 1: table S7). The Partial Dependence plots (Figs 6, 7) evidence that such relationship varies amongst areas of *Carpobrotus* spp. expansion (EXP_{CAR}) and reduction (RED_{CAR}). The RF models and their setup (*Mtry*, *Ntree*, *MNS*) provided an adequate description of the landscape dynamics of *Carpobrotus* spp. invasion, including changes in the spatial pattern of the surrounding landscape (ΔLM_{class} and ΔLM_{land}; Suppl. material 1: table S8). The spatial-temporal dynamics of *Carpobrotus* spp. patches ($\Delta PLAND_{CAR}$, ΔPD_{CAR}, ΔED_{CAR}, $\Delta AREA\_MN_{CAR}$) are significantly correlated with coastal

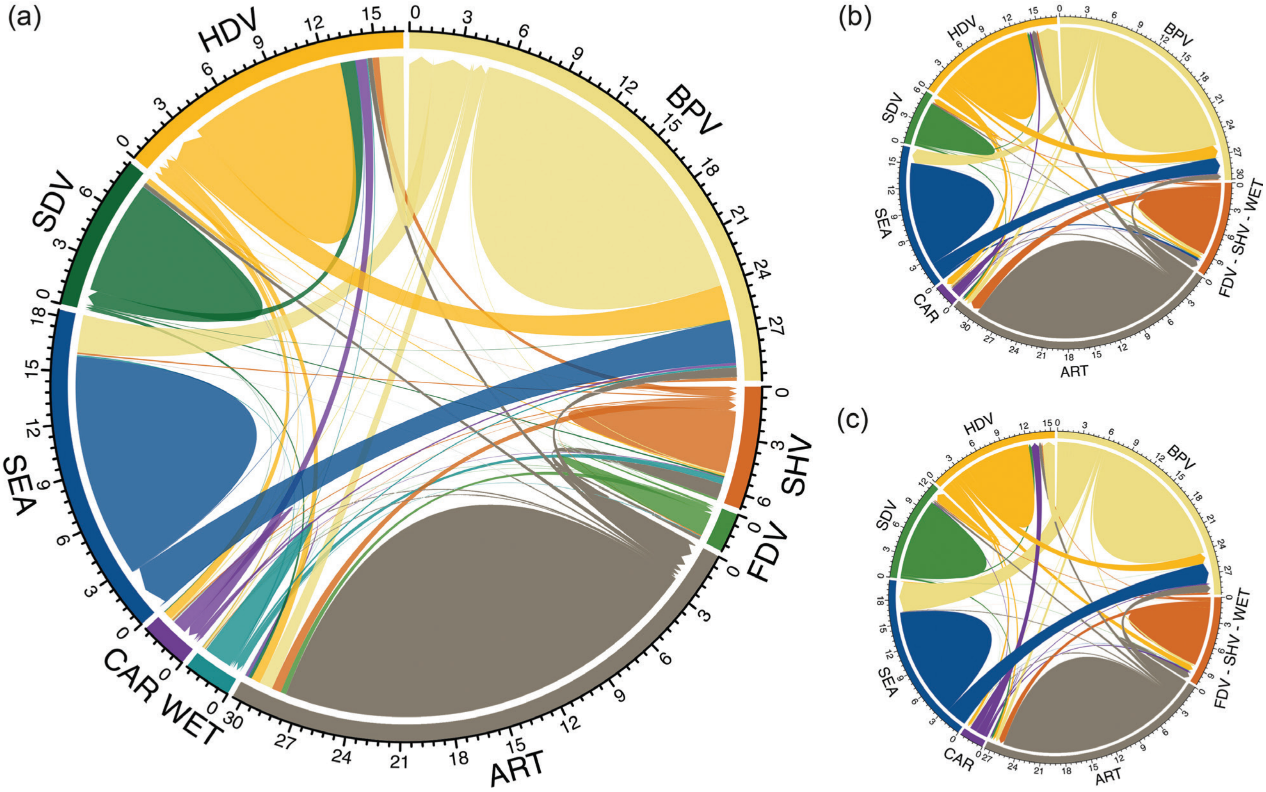
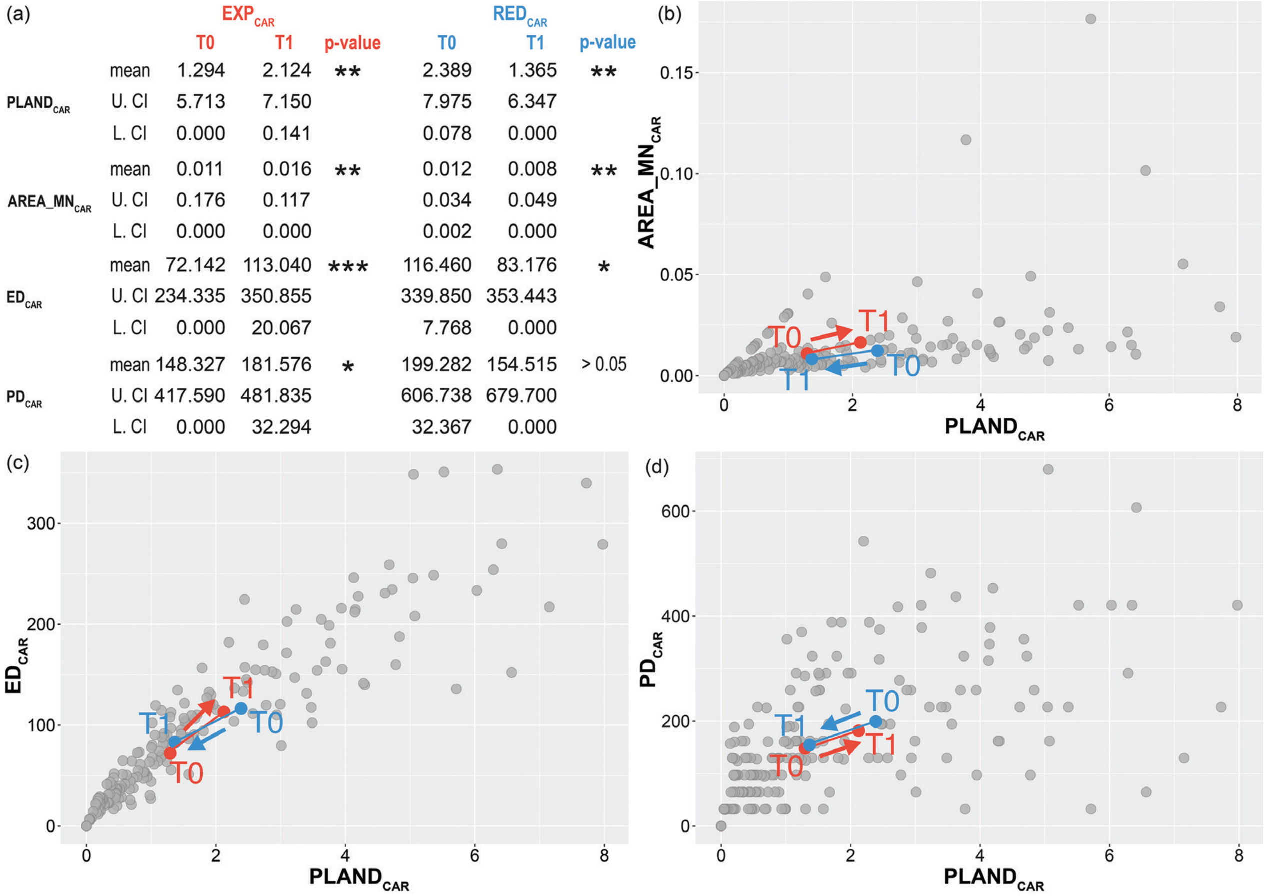


Figure 4. Chord diagrams for: **a** all coastal tracts **b** tracts with *Carpobrotus* spp. expansion (EXP_{CAR}) and **c** tracts with *Carpobrotus* spp. reduction (RED_{CAR}). The chord diagrams summarise the percentage (%) of each land-cover class in T0 (outer ring) that changed into another class to T1. The size and the direction of arrows represent transitions to other classes in T1. For example, an increase of HDV from BPV in T1 considering all coastal tracts. The proportion (%) of each land-cover class that remained stable over time is represented by the internal coloured circle. Land-cover classes: artificial areas (ART), beach with pioneer annual vegetation (BPV), herbaceous dune vegetation (HDV), shrub dune vegetation (SDV), Forest and woody dune vegetation (FDV), semi-natural herbaceous vegetation (SHV), wetland (WET), Tyrrhenian sea (SEA), *Carpobrotus* spp. (CAR).

dune landscape changes (with a minimum R^2 of 0.405 for the $EXP\_\Delta PD_{CAR}$ model and 0.595 for the $RED\_\Delta PLAND_{CAR}$ model; Figs 6, 7).

In coastal tracts of IAP expansion (EXP_{CAR}), the landscape change variables ($RF\_\Delta LM$) that better explain *Carpobrotus* spp. evolution ($EXP\_\Delta LM_{CAR}$) are the size and the shape complexity of herbaceous dune vegetation, of the sea and of artificial areas ($\Delta AREA\_MN_{HDV}$, $\Delta AREA\_MN_{SEA}$, $\Delta AREA\_MN_{ART}$, ΔED_{HDV}, ΔED_{SEA}, ΔED_{ART}; Fig. 6). A higher increment of *Carpobrotus* spp class cover ($\Delta PLAND_{CAR}$) occurs in correspondence with the reduction of sea-class surface ($\Delta AREA\_MEAN_{SEA} \approx$ -0.25 to -0.50), with increasing contacts with artificial infrastructures ($\Delta ED_{ART} \approx$ 100 to 250) and with intermediate reduction in the number of natural dune vegetation patches ($\Delta PD_{HDV} \approx -150$). We also registered an increase in the number of invaded patches (ΔPD_{CAR}, Fig. 6b) in correspondence with increasing edge length of beach-pioneering vegetation ($\Delta ED_{BPV} \approx$ 100 to 200) and of herbaceous vegetation cover ($\Delta ED_{HDV} \approx$ 100 to 200) and the reduction of herbaceous vegetation cover ($\Delta PLAND_{HDV} \approx$ less to -10). Concerning the increase of invaded patches edge length (ΔED_{CAR}, Fig. 6c), it tends to occur at increasing edge length of dune vegetation ($\Delta ED_{HDV} \approx$ 100 to 200) and of artificial areas ($\Delta ED_{ART} \approx$ greater than 200), as well as at decreasing cover of sea class ($\Delta AREA\_MEAN_{SEA} \approx$ less to -0.25). As observed with invasion cover ($\Delta PLAND_{CAR}$), also the dimension



(a)

		EXP_{CAR}			RED_{CAR}		
		T0	T1	p-value	T0	T1	p-value
$PLAND_{CAR}$	mean	1.294	2.124	**	2.389	1.365	**
	U. CI	5.713	7.150		7.975	6.347	
	L. CI	0.000	0.141		0.078	0.000	
$AREA\_MN_{CAR}$	mean	0.011	0.016	**	0.012	0.008	**
	U. CI	0.176	0.117		0.034	0.049	
	L. CI	0.000	0.000		0.002	0.000	
ED_{CAR}	mean	72.142	113.040	***	116.460	83.176	*
	U. CI	234.335	350.855		339.850	353.443	
	L. CI	0.000	20.067		7.768	0.000	
PD_{CAR}	mean	148.327	181.576	*	199.282	154.515	> 0.05
	U. CI	417.590	481.835		606.738	679.700	
	L. CI	0.000	32.294		32.367	0.000	

Figure 5. Comparison of *Carpobrotus* spp. pattern metrics ($PLAND_{CAR}$, PD_{CAR}, ED_{CAR}, $AREA\_MN_{CAR}$) over time (from T0 to T1) in tracts of expansion (red: EXP_{CAR}) and reduction (blue: RED_{CAR}). **a** Kruskal-Wallis comparison of means and the respective confidence intervals (upper – U. CI, lower – L. CI; * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001) **b–d** report the trajectory analysis of *Carpobrotus* spp. patches area ($AREA\_MN_{CAR}$), edge density (ED_{CAR}) and patch density (PD_{CAR}) in relation to overall *Carpobrotus* spp. cover ($PLAND_{CAR}$). Grey dots represent the observed values of pattern metrics, coloured dots the arithmetic mean (mean of $PLAND_{CAR}$, PD_{CAR}, ED_{CAR}, $AREA\_MN_{CAR}$) in each date (T0 and T1) in EXP_{CAR} and RED_{CAR} and arrows indicate the direction of temporal change.

of invaded patches ($\Delta AREA\_MN_{CAR}$, Fig. 6d) tend to increase in correspondence with the reduction of sea-class surface ($\Delta AREA\_MEAN_{SEA} \approx$ less to -0.25), with increasing or decreasing urban cover ($0.30 < \Delta AREA\_MN_{ART} < -0.30$) and stable herbaceous vegetation edge length ($ED_{HDV} \approx 0$).

Partial dependence plots for less important change variables in EXP_{CAR} RF models are provided in Suppl. material 1: figs S12–S15.

In coastal tracts of IAP reduction (RED_{CAR}), the landscape change variables (RF_ΔLM) that explain *Carpobrotus* spp. contraction ($RED\_\Delta LM_{CAR}$; Fig. 7) include both: class metrics of the main cover classes (e.g. ΔLM_{HDV}, ΔLM_{ART}, ΔLM_{SEA} and ΔLM_{BPV}) and landscape metrics (**Δ**SIDI). Stronger contraction of *Carpobrotus* spp class cover ($\Delta PLAND_{CAR}$; Fig. 7a) occurs in correspondence with changing beach pioneer vegetation edges length (decreasing $\Delta ED_{BPV} \approx$ -200 to -300 or increasing $\Delta ED_{BPV} \approx 100$), with increasing herbaceous vegetation cover ($\Delta PLAND_{HDV} \approx 10$), as well as with a consistent increase in artificial areas edges ($\Delta ED_{ART} \approx$ 100 to 200). We also registered a decrease in the number of invaded patches (ΔPD_{CAR}, Fig. 7b) in correspondence with decreasing class metrics of herbaceous vegetation as edge length ($\Delta ED_{HDV} \approx -200$) and number of patches ($\Delta PD_{HDV} \approx$

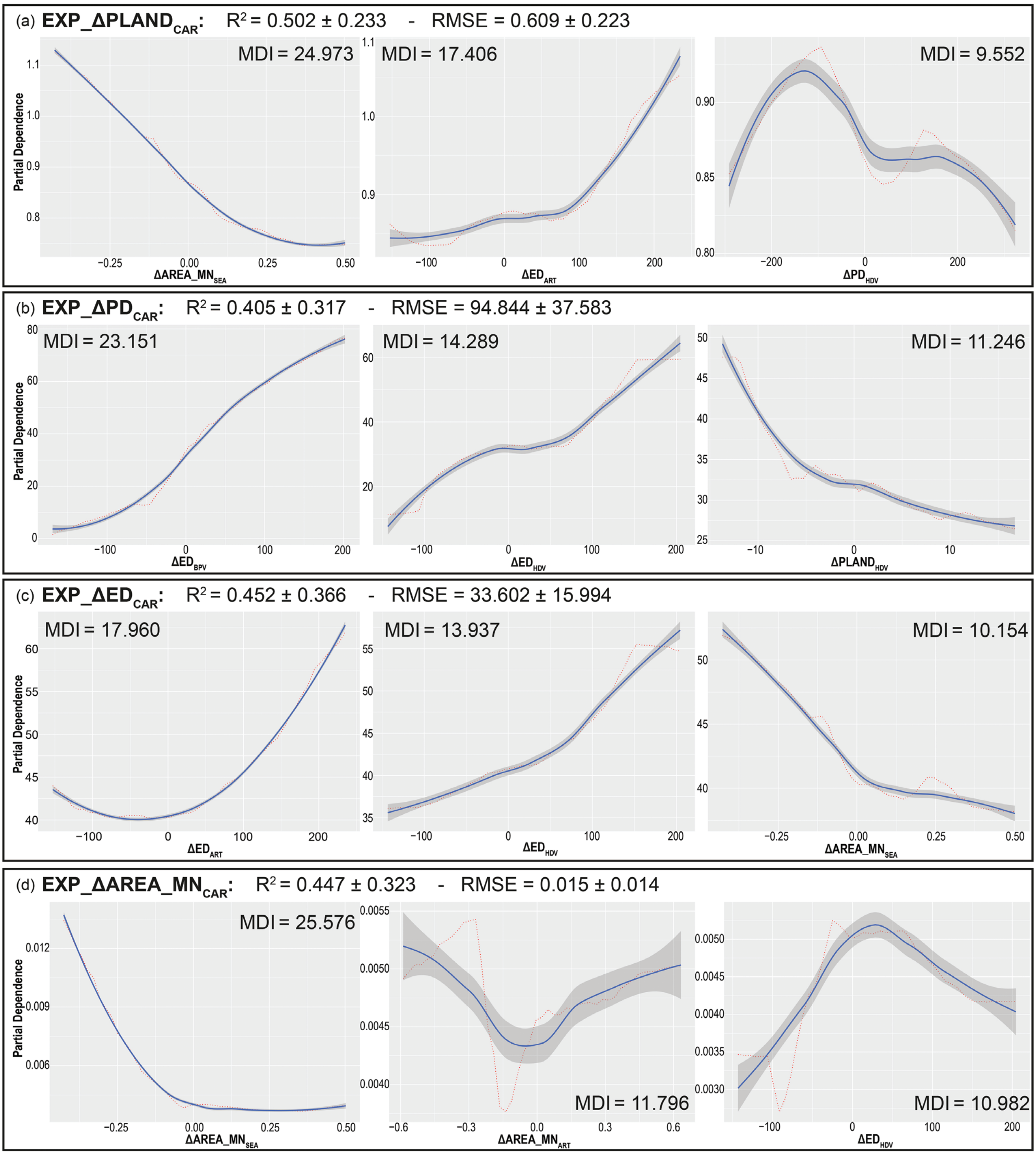


Figure 6. Partial dependence plots (PDP) using linear smoothing of the most important variables (over of 40% in cumulate importance) on the RF models for areas of alien expansion (EXP_ΔLM$_{CAR}$). **a** EXP_ΔPLAND$_{CAR}$ **b** EXP_ΔPD$_{CAR}$ **c** EXP_ΔED$_{CAR}$ **d** EXP_ΔAREA_MN$_{CAR}$. Red dotted lines represent raw PDP curves. The importance of each variable is indicated by the Mean Decrease Importance (MDI) value. For land-cover classes, see Table 1 and for pattern metrics description, see Table 2.

-100 to -200) and the increment of urban class cover (AREA_MN$_{ART}$ > 0). Regarding the decrease in invaded patch edge length (ΔED$_{CAR}$, Fig. 7c), it coincides with the reduction in herbaceous vegetation cover (ΔPLAND$_{HDV}$ ≈ -10) and edge density (ΔED$_{HDV}$ ≈ -200 to -250) and with the simplification of landscape diversity (ΔSIDI ≈ -0.025). A consistent reduction on *Carpobrotus* spp. class patch

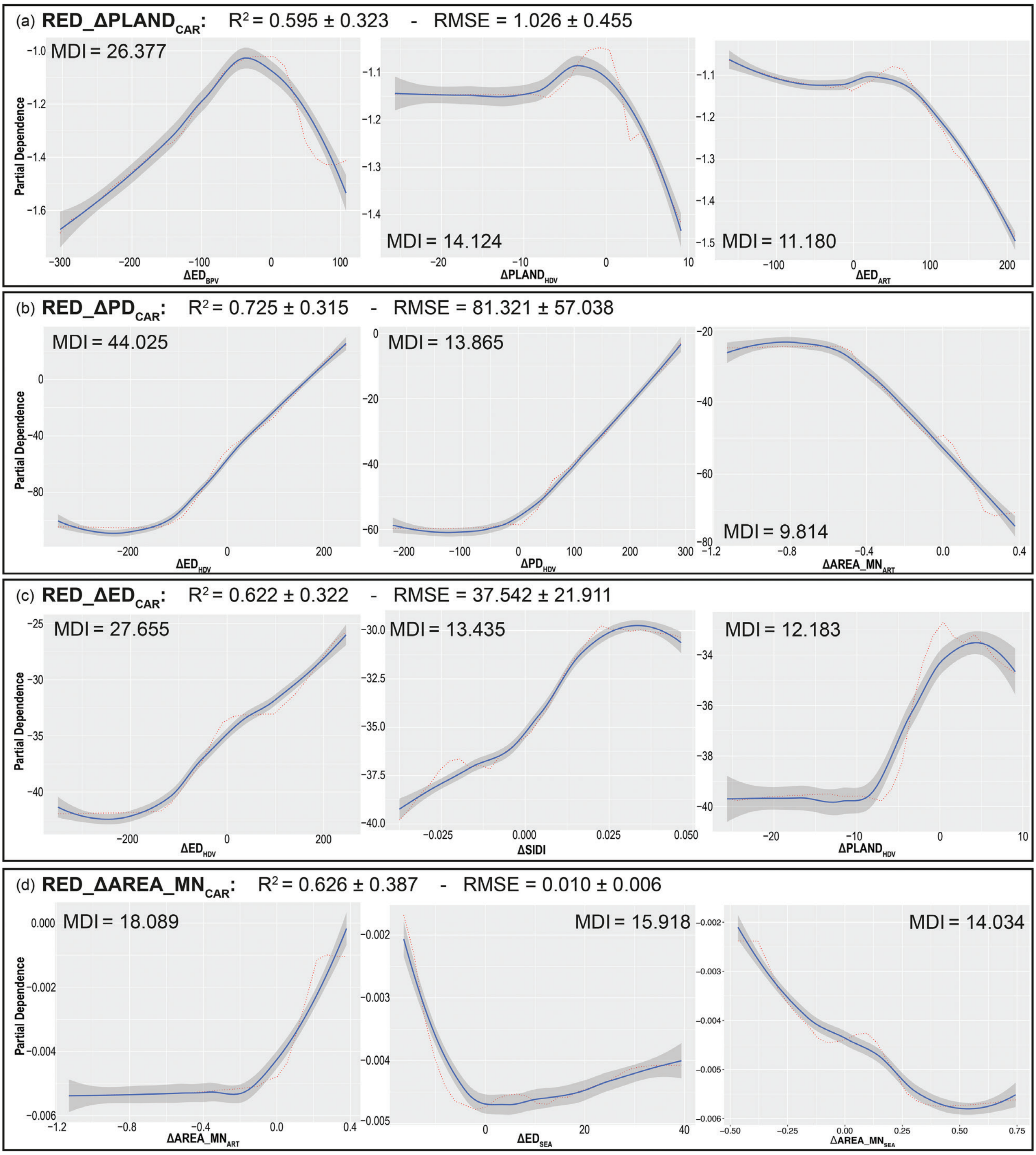


Figure 7. Partial dependence plots (PDP) using linear smoothing of the most important variables (over of 40% in cumulate importance) on the RF models for areas of alien reduction (RED_ΔLM$_{CAR}$). **a** RED_ΔPLAND$_{CAR}$ **b** RED_ΔPD$_{CAR}$ **c** RED_ΔED$_{CAR}$ **d** RED_ΔAREA_MN$_{CAR}$). Red dotted lines represent raw PDP curves. The importance of each variable is indicated by the Mean Decrease Importance (MDI) value. For land-cover classes, see Table 1 and for pattern metrics description, see Table 2.

size (ΔAREA_MN$_{CAR}$) seems related with increasing values of SEA class metrics as wider surface (ΔAREA_MN$_{SEA}$ > 0.25) and stable edges (ΔED$_{SEA}$ ≈ 0), as well as with the reduction of artificial surfaces (ΔAREA_MN$_{ART}$ < 0). The dependence trends of other change variables in RED$_{CAR}$ areas are reported in Suppl. material 1: figs S16–S19 for RED_ΔLM$_{CAR}$ models.

Discussion

We analysed the spatiotemporal changes in *Carpobrotus* spp. invasion and its relationship with landscape composition and configuration in tracts of expansion and reduction over time (from T0 to T1) in Mediterranean coastal dunes. Our results highlighted the importance of using satellite and aerial imagery with minimal co-registration errors to effectively analyse the temporal dynamics of coastal dune landscapes and invasion process (Talavera et al. 2022). Landscape changes have concerned approximately 20% of the total area with most changes involving significant shifts between neighbouring patches. Such shifts are quite common on highly dynamic ecosystems such as coastal dunes (Acosta et al. 2003; Drius et al. 2013). As on most of the Mediterranean seashores, urban areas and infrastructures (ART) resulted in being quite extensive (≈ 28%) and expanded over time at the expense of natural formations, such as beach and herbaceous dune vegetation (Malavasi et al. 2013).

Relationship between *Carpobrotus* spp. invasion and landscape dynamics in expansion tracts

Carpobrotus spp. tends to expand in coastal zones characterised by stable accreting seashore (where the SEA class area remains stable or diminishes) and by increasing urban surfaces (where ART class patches increase). The expansion of *Carpobrotus* spp. on coastal tracts experiencing seashore accretion and stability may be likely related with the fact that these seashore processes promote the development of herbaceous dune habitats (Bazzichetto et al. 2020) which are known to be especially susceptible for colonisation by invasive alien plants (IAPs) like *Carpobrotus* spp. (Carranza et al. 2011). Indeed, *Carpobrotus* spp. primarily expands by displacing herbaceous natural vegetation, confirming the high vulnerability of this natural habitat to IAP invasions. Our results gave new bi-temporal evidence of the habitat preference of *Carpobrotus* spp. to herbaceous habitats postulated in the past based on static data (e.g. Carranza et al. (2011); Bazzichetto et al. (2018a)) or using a diachronic analysis (Sperandii et al. 2018). The observed increase of IAP's and urban-patches cover, is likely attributable to the role of artificial areas as a source of non-native species (Carranza et al. 2010) and provides bi-temporal evidence in support of the propagule pressure theory.

The trajectory analysis evidenced a significant rise of all the considered *Carpobrotus* spp. spatial metrics denoting a consistent process of invasion. Indeed, the extension of invaded areas increased (greater $PLAND_{CAR}$) and *Carpobrotus* spp. tended to be distributed in more numerous (PD_{CAR}), larger ($AREA\_MN_{CAR}$) and irregularly-shaped patches (ED_{CAR}). As observed in other colonisation processes such as forest regrowth (Malavasi et al. 2018b), the increase in the number of patches may denote the emergence of a new nucleus of colonisation, while the enlargement of patch area may indicate the growth of already established invaded points. On the other hand, the observed increase in edge length may indicate the maturity of invasion, with *Carpobrotus* spp. patches adopting the typical long-shaped pattern along the seashore of the invaded natural herbaceous dune vegetation (Carranza et al. 2010).

The pattern of expansion of *Carpobrotus* spp. (ΔLM_{CAR}) is significantly associated with landscape dynamics (ΔLM_{land}). Amongst the landscape change variables that best explain *Carpobrotus* spp. expansion, the seashore stability or accretion (ΔSEA close or higher than 0), the increasing surface and edges of urban areas (ΔART) and the size and shape complexity of herbaceous dune vegetation, emerge as particularly influential factors. The observed increase in *Carpobrotus* spp. cover, patch size and edge length in stable or accreting coastal dunes (e.g. sea class surface reduction), highlights the strong correlation between the presence of dunes, their stability and the heightened susceptibility of landscapes to *Carpobrotus* spp. invasion. Our bi-temporal analysis-based results provide additional evidence supporting the vulnerability of dunes, a principle previously suggested by invasion risk models using the distance to the shoreline as a surrogate of coastal dune zonation (Bazzichetto et al. 2018a, b).

In tracts registering IAP expansion, *Carpobrotus* spp. patches become larger and more irregularly shaped in correspondence with an increase in the cover and edge length of artificial areas. This is likely linked to the role of built-up and urban structures, as well as artificial edges in driving invasion processes (Malavasi et al. 2014; Bazzichetto et al. 2018a, b). In these tracts, urban areas may play a double role: an important source of invasive alien propagules and key ecological corridors assuring landscape connectivity for the colonisation of new areas (Boscutti et al. 2022; Lozano et al. 2023). Our results also evidenced that altered coastal landscapes (e.g. detrimentally changed dune vegetation composition and configuration features) might undergo further modification due to the colonisation and spread *Carpobrotus* spp. (Malavasi et al. 2014). The creation of new artificial corridors (e.g. infrastructures) on fragmented coastal landscapes could aid the invasion process with detrimental effects on dune integrity and natural habitats. As observed for other IAPs, the registered landscape trends may be compatible with further growth of *Carpobrotus* spp. that conforming dense monospecific carpets could alter coastal dune landscape composition and configuration (Kozhoridze et al. 2022). The colonisation and expansion trends pinpoint the need for planning and implementing dedicated measures to contain and prevent further *Carpobrotus* spp. expansion at the expense of herbaceous dune vegetation classes which is urgent as it includes several habitats of European conservation concern (EU-2110: embryonic shifting dunes, EU-2120: shifting dunes along the shoreline with *Ammophila arenaria;* EU-2210: *Crucianellion maritimae* fixed-beach dunes, EU-2230: *Malcolmietalia* dune grasslands). These measures encompass both reducing and mitigating dune fragmentation and degradation processes, as well as monitoring activities in the most susceptible landscape elements to aid the implementation of effective prevention and early warning actions.

Relationship between *Carpobrotus* spp. invasion and landscape dynamics in reduction tracts

Within the coastal tracts of *Carpobrotus* spp. contraction ($\mathrm{RED}_{\mathrm{CAR}}$), landscape spatial-temporal characteristics resulted in being quite dynamic with an intense seashore erosion (SEA class area increase) that constrained the coastal dune zonation to small areas and that curtailed the spatial complexity of overall the natural mosaic (Doody 2004, 2013). In such areas, the SEA category is replaced by beach pioneer vegetation (BPV), that, in turn, substitutes herbaceous dune vegetation (HDV).

The decline in *Carpobrotus* spp. cover on coastal tracts experiencing seashore erosion may be attributed to the erosion's detrimental impact on habitats suitable for the invasive plant's colonisation, such as beach and herbaceous vegetation (Carranza et al. 2011; Bazzichetto et al. 2018a). While this hypothesis has primarily been examined in the context of management activities aimed at eradicating *Carpobrotus* spp., further research is needed to validate its broader applicability (Chenot et al. 2018).

In these coastal tracts, *Carpobrotus* spp. tends to be substituted by herbaceous dune vegetation and its pattern in T1 resulted in being simplified into smaller and regularly-shaped patches with respect to IAP pattern on the T0. As evinced by trajectory analysis, the temporal reduction of invaded areas (lower $PLAND_{CAR}$) with *Carpobrotus* spp patches distributed on smaller ($AREA\_MN_{CAR}$) and regularly-shaped patches (ED_{CAR}) suggest that *Carpobrotus* spp. is undergoing fragmentation. As observed in other fragmentation process (Wang et al. 2014; Carranza et al. 2015), the reduction of *Carpobrotus* spp. area into patches may denote the retreat and disappearance of invaded areas, while the reduction of patch size may indicate the contraction of the remnant invaded points.

The reduction pattern of *Carpobrotus* spp. (ΔLM_{CAR}) is linked to landscape changes (ΔLM_{land}), specifically those concerning coastal erosion (SEA) and land take (ART). Both processes contribute to the "squeezing" of dune zonation, compressing HDV and BPV communities into simplified small relict areas (Martínez et al. 2014; Gilby et al. 2021). In these tracts, the coastal "squeeze" process reduces suitable land space for coastal dune ecosystems and, consequently, the possibility to maintain their essential functions (Martínez et al. 2014). Under these "squeeze" conditions, only hard structures, such as building and human infrastructures, remain, while both *Carpobrotus* spp. patches and native vegetation tend to diminish and eventually disappear.

Within one decade, *Carpobrotus* spp. registered a decline on cover, patch size and edge length which occurred together with the simplification of natural and semi-natural land-cover classes (e.g. the reduction on cover, edge length and aggregation of herbaceous and pioneer dune vegetation) and overall landscape diversity (SIDI). Moreover, this reduction coincides with increased artificialisation (expanding urban areas in terms of area and edge length) and seashore erosion (expansion of sea area and edge length). The widespread decrease in landscape diversity and the deterioration of dune integrity (Acosta et al. 2003; Drius et al. 2013), combined with the fragmentation (*sensu* Wang et al. (2014)) observed in the invasion pattern of *Carpobrotus* spp., suggest a substantial influence of both abiotic factors (seashore erosion) and human-driven forces (urbanisation) on shaping the entirety of the coastal dune mosaic, encompassing both natural and invaded areas.

Conclusion

Our results highlight the significant potential of temporal analysis for monitoring invasive alien plants trends in complex dynamic mosaics like Mediterranean coastal systems. This temporal analysis of coastal composition and configuration has provided evidence of various processes: the stability of coastal dunes and the expansion of urban areas, that increase landscape vulnerability to invasions, as well as erosion and coastal "squeeze", which negatively impact invasion dynamics. These findings corroborate earlier conclusions from literature which were largely based on static data and emphasise the value of dynamic analyses for understanding and managing coastal landscapes.

The adopted temporal mapping and modelling approach effectively captures various changes in plant invasion, correlating them with ongoing landscape processes. This not only provides enhanced monitoring tools, but also advances our understanding of invasion processes at a landscape scale, meeting the objectives outlined in the Convention on Biological Diversity and in the Regulation EU no. 1143/2014. Indeed, our results provide valuable insights for addressing management plans tailored to specific landscape contexts. For instance, in coastal tracts experiencing seashore accretion and urban growth, *Carpobrotus* spp. colonises stable coastal areas, displacing native herbaceous dune vegetation. In these tracts, urban expansion and the availability of open sand with herbaceous dune vegetation serve as key drivers of invasive alien plants (IAPs) proliferation. Consequently, targeted monitoring activities should be prioritised, focusing on herbaceous dune vegetation, to detect, control and eradicate *Carpobrotus* spp. patches. Conversely, the reduction of *Carpobrotus* spp. is observed in seashores affected by erosion and subjected to the “squeeze” process. This reduction leads to smaller, simplified patches, indicating fragmentation and the eventual disappearance of invaded areas. Therefore, management actions and projects aimed at mitigating the coastal erosion and the “squeeze” process (Leo et al. 2019) and preventing the potential re-invasion of *Carpobrotus* spp. should be implemented in reduction tracts.

The proposed methodology could be further extended to other datasets to calculate invasion trends through a fully temporal assessment. Additionally, new multi-temporal analysis may be adopted to evaluate the effectiveness of IAP management actions over time and support the implementation of adaptive management strategies. The use of temporal maps and data offers a cost-effective solution for monitoring IAPs across broad geographic areas, addressing the resource constraints often associated with field data collection campaigns. Therefore, we strongly advocate for the adoption of temporal landscape analysis as a monitoring tool to bridge the gap between scientific knowledge and IAP management practices. This approach provides tailored and efficient solutions for environmental managers, facilitating more informed and effective decision-making.

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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

Supplementary data

Authors: Flavio Marzialetti, Giacomo Grosso, Alicia Teresa Rosario Acosta, Marco Malavasi, Luigi Cao Pinna, Marcelo Sternberg, Sharad Kumar Gupta, Giuseppe Brundu, Maria Laura Carranza

Data type: docx

Explanation note: Results of non-linear relations between invasion dynamics and landscape changes (**figs S1–S8**). Confusion matrices of land cover maps in 2022 (**table S1**) and in 2012 (**table S2**). Transition matrices of land cover changes in all study area (**table S3**), in areas with expansion of *Carpobrotus* spp. invasion (**table S4**), and in areas with reduction of *Carpobrotus* spp. invasion (**table S5**). Trajectory analisis of Artificial class pattern metrics (**fig. S9**). Result of Variance Inflation Factor (VIF, **table S6**). Results, performances, and variable importances of Random Forest models (**table S7**). Partial dependence plots (PDP) of random forest models using linear smoothing from the 4th to the 13th variables in order of importance (**figs S10–S17**).

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Research Article

On the path to cosmopolitanism: the continuing geographic expansion of *Caprella mutica* (Crustacea, Amphipoda)

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Abstract

Human activities have been transporting caprellid amphipods (or “skeleton shrimps”) across the oceans for many decades. As a result, some caprellid amphipods now are among the most widespread non-indigenous species in many different coastal regions of the world. The global spread of these species is still ongoing in some cases, such as that of the successful invader *Caprella mutica* Schurin, 1935. Here, we report on the arrival of *C. mutica* in South America and modelled its environmental niche based on its current global distribution in order to evaluate future expansion risks. The species distribution model confirmed high occupancy probabilities for already invaded areas of Europe and North America with generally lower probabilities in the southern hemisphere and mean sea surface temperature as best predictor. Further, the model suggested that our discovery of *C. mutica* in northern Chile was made in a region that is less favorable for this species, while occupancy probabilities increased further south. Given the invasion history of *C. mutica* in other marine regions of the world and the more favorable oceanographic conditions, a further spread of this invader southwards along the South American Pacific coast seems very likely.

Key words: Biofouling, biological invasion, marine dispersal, marine shipping, non-indigenous species

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Introduction

Many marine species have been transported by human activities across the oceans to other coastal regions where they established as non-indigenous species (NIS). This human-mediated spread creates recent ‘neocosmopolitan’ distributions of species (i.e. extensive geographical ranges through anthropogenic dispersal) among coastal benthic habitats around the world (Darling and Carlton 2018). Crustaceans, including barnacles, shrimps, crabs and peracarids are among some of the most ubiquitous NIS worldwide (Ruiz et al. 2011; Bailey et al. 2020). While large decapod species have likely been transported in ballast water of cargo ships (Rodríguez and Suárez 2001), many smaller amphipod species are assumed to have been dispersed as stowaways on ship hulls or with aquaculture activities (e.g. Marchini and Cardeccia 2017; Albano and Obenat 2019; Martínez-Laiz et al. 2019; Guerra-García et al. 2023). Many of these amphipod NIS are now cosmopolitan species but their former native ranges cannot always be determined (Beermann et al. 2020; Martínez-Laiz et al. 2021). This is because their invasion history is often ancient, sometimes dating back hundreds of years to the first circumglobal expeditions (Darling and Carlton 2018; Beermann et al. 2020).

Interestingly, caprellid amphipods are among the most widespread neocosmopolitan species that have become NIS in many different regions of the world (Cabezas et al. 2010; Ros et al. 2016; Marchini and Cardeccia 2017; Martínez-Laiz et al. 2021). For example, *Caprella scaura* Templeton, 1836 successfully established dense populations all around the globe in warm-temperate to tropical waters where it thrives in fouling communities on man-made substrata (e.g. Guerra-García et al. 2011; Martínez-Laiz et al. 2021). At least in southern Europe, it competes with another cosmopolitan caprellid, *Caprella equilibra* Say, 1918, which can occur in the same habitats (Foster et al. 2004; Ros et al. 2015; Marchini and Cardeccia 2017).

In recent decades, many new species introductions had been recognized shortly after their initial arrival in new regions. The invasion history of these newly introduced species could be documented, allowing to better understand the mechanisms of transport and expansion into new regions. One of these cases of ongoing invasions is the caprellid amphipod *Caprella mutica* Schurin, 1935. Native to North-East Asia, it was described from the Sea of Japan (Schurin 1935; Vassilenko 1967; Arimoto 1976) before it established populations on many coasts outside its native range within a relatively short time (Boos et al. 2011; Marchini and Cardeccia 2017). The successful establishment of amphipod NIS in an area can result in a displacement of ecologically similar native species (e.g. Dick 1996; Dick et al. 1999). Correspondingly, mass occurrences of invasive *C. mutica* have been linked to a scarcity of native caprellids in the same habitats such as *Caprella linearis* (Linnaeus, 1767) in the North East Atlantic (e.g. Coolen et al. 2016). This could be due to direct interference competition between the invader and native caprellids (Shucksmith et al. 2009) although differential habitat demands (Coolen et al. 2016) may facilitate a spatial segregation and thus resource partitioning (Schoener 1986). Based on its known habitat preferences and environmental tolerances, Boos et al. (2011) speculated on a future range expansion of *C. mutica* to several coastlines around the globe where it had not been reported yet, but environmental conditions seemed favorable. Among these potentially vulnerable regions were the Pacific and Atlantic coasts of South America.

In South America, only few amphipod NIS have been reported so far, and they are often underrepresented or even completely absent from NIS inventories (e.g. Ferreira et al. 2009; Cárdenas-Calle et al. 2019; Carlton et al. 2019; Teixeira and Creed 2020; Zambrano and Ramos 2021; Rodríguez-Gavilanes et al. 2024), or their status is not well known and many species are categorized as cryptogenic (e.g. Orensanz et al. 2002). An exception to this general pattern is the Argentinian coast, where 18 amphipod NIS (including introduced and cryptogenic species) have been reported, among them three caprellid species (Schwindt et al. 2020). The cosmopolitan species *Caprella danilevskii* Czerniavsky, 1868, *C. equilibra* and *C. scaura* have been reported widely from the Atlantic and Pacific coasts of South America (Guerra-García and Thiel 2001; Díaz et al. 2005; Cunha et al. 2018; Chunga-Llauce et al. 2023b). Furthermore, the caprellid *Paracaprella pusilla* Mayer, 1890 also has recently been reported as NIS from locations along the Pacific coast (Alarcón-Ortega et al. 2015; Ros et al. 2016; Alfaro-Montoya and Ramírez Alvarado 2018; Chunga-Llauce et al. 2022), suggesting that caprellid invasions might not go entirely undetected along the coasts of South America.

Few amphipod NIS have been reported for the coasts of Chile. In their review of marine NIS of the southeastern Pacific, Castilla and Neill (2009) listed no amphipod NIS for the coasts of Chile and Peru. However, two frequently occurring species of the genus *Jassa* Leach, 1814 have been confirmed to be NIS in Chile and South America in general (Beermann et al. 2020). Further, the cosmopolitan *Monocorophium acherusicum* (A. Costa, 1853) has been recorded from fouling communities in Chile (Pérez-Schultheiss 2009; Thiel and Hinojosa 2009), and several other amphipod species have been suggested as NIS for Chile (Marchini and Cardeccia 2017). The littoral caprellid fauna of Chile has been relatively well-characterized, based on extensive sampling in benthic and artificial habitats at 30°S in Coquimbo in northern central Chile (Guerra-García and Thiel 2001). A follow-up study then surveyed caprellids from harbor and aquaculture buoys along the Chilean coast between 18°S and 41°S, confirming the same species as previously reported for Coquimbo with the exception of the species *Deutella venenosa* Mayer, 1890, which seemed to be restricted to Coquimbo (Thiel et al. 2003). Since then, the caprellid fauna of Chile and Coquimbo in particular has been screened occasionally, also checking for potential newcomers (Astudillo et al. 2009; Rech et al. 2023).

The aim of this study is to (i) report the arrival of the successful invader *Caprella mutica* in South America, (ii) compile recent surveys of the caprellid fauna from several regions in South America, (iii) synthesize information about the current distribution of *Caprella mutica*, and (iv) build a species distribution model of *Caprella mutica* to evaluate the future expansion risk of this species along the South American coasts.

Material and methods

Sampling

Caprellid specimens were repeatedly collected at the same site with fouling assemblages from floating docks and on mooring lines of the aquaculture concessions of the Universidad Católica del Norte (UCN) in Bahía La Herradura in Coquimbo, Chile (29°57'58.4"S, 71°21'12.9"W) on August 30th, 31st and September 9th 2022. Several culture lines for scallop aquaculture are established in the concession of UCN, where lantern-nets are suspended from longlines (Bakit et al. 2024). These artificial structures host extensive fouling communities (Dumont et al. 2009) providing habitat to many mobile organisms (including several species of caprellid amphipods) (Astudillo et al. 2009). For the sampling procedure, the buoys and longlines were lifted up from a boat, and the fouling biomass was scraped from these artificial substrata and brought to the lab (approx. transport time: 10 min). For each sampling we collected an approximate volume of about 10 l fouling biomass, which included seaweeds, hydrozoans, bryozoans, tunicates, mussels and other sessile organisms (for species inventory see e.g. Astudillo et al. 2009). Additional material was obtained from samples collected the same way and at the same site on 23 June and 7 July 2023.

In the laboratory, the fouling organisms were immediately placed in large trays (approximately 20 cm x 30 cm surface area) with seawater, and the material was sorted alive. No signs of predation in the samples were observed during the procedure. All amphipods were retrieved and carefully inspected under a dissecting microscope. Caprellid amphipods were identified to the lowest taxonomic level, and counted. Voucher material was fixated in ethanol and deposited in the collection of the UCN.

During the years 2004 to 2023, caprellid amphipods were collected annually for the Invertebrate Zoology laboratories in the Marine Biology program of the Marine Science Faculty in Coquimbo (30°S). For these courses, usually a few hundred live caprellid individuals were brought to the teaching laboratory (on seaweeds, bryozoans and hydrozoans). The collection of caprellid amphipods was conducted in a very similar way as described above by sampling extensive amounts of fouling organisms with the associated caprellid amphipods. The samples were collected a few hours before the course, transported to the nearby lab, and maintained alive for students to observe and document the morphology and behavior of the caprellids. Students quantified the ventilation movements of ovigerous caprellid females and had to identify the particular species for which they recorded these behaviors using Guerra-García and Thiel (2001); the species identifications were usually checked by the course instructors.

Review of caprellid studies

In order to characterize the recent survey efforts focusing on the caprellid fauna in Central and South America, we searched the literature using the Web of Science and GoogleScholar. The keywords "Caprella" and "amphipod" were linked with the names of all Central and South American countries. In order to identify additional studies, all studies on caprellids that were published after 2000 were carefully examined for cross-citations. The recovered references were then scanned to identify those that reported on caprellid surveys in their regions or countries. These studies typically included species inventories that were based on targeted samplings of the caprellid fauna. All studies were conducted by invertebrate zoologists, often including amphipod or even caprellid specialists, who were very familiar with the taxonomic literature and species identifications. The investigations focused on shallow habitats up to approximately 20 m water depth, including fouling communities (e.g. Nunez Velazquez et al. 2017; Chunga-Llauce and Pacheco 2021; Chunga-Llauce et al. 2022) and macrophyte or animal reefs (Díaz et al. 2005; Alarcón-Ortega et al. 2017; Cunha et al. 2018). Usually the authors sampled several sites within their study region, where individual sites had distances of a few to > 100 km between them. Most studies covered one or maximally two ecoregions (*sensu* Spalding et al. 2007). References that focused only on the population or reproductive biology of selected caprellid species were not included.

For comparative purposes, we extracted presence/absence data from each respective study, which is common practice in biodiversity reviews of specific groups or regions (see e.g. Gallardo and Penchaszadeh 2001; Cárdenas-Calle et al. 2020; Durand et al. 2024). Only records on species-level were considered in the current data consolidation. The similar approaches used by all examined studies allowed for direct comparison in the context of the current overview.

Species distribution model (SDM) of *Caprella mutica*

Worldwide georeferenced occurrences for *C. mutica* were downloaded and curated from the Global Biodiversity Information Facility (GBIF, www.gbif.org; downloaded on 06 September 2023). The database was augmented by an exhaustive literature search and further published records were added (i.e. derived from:

Schurin 1935; Vassilenko 1967; Arimoto 1976; Locke et al. 2007; Ashton et al. 2008a; 2008b; Willis et al. 2009; Hosono 2011; Almón et al. 2014; Collin and Johnson 2014; Coolen et al. 2016; Peters and Robinson 2017; Heo et al. 2020; Lavrador et al. 2024). A total of 1388 occurrences of *C. mutica* were used for the model (excluding the current presence in Chile reported here; Fig. 1). The occurrences were thinned to reduce sampling biases (Aiello-Lammens et al. 2015), leaving only one presence per grid cell (0.08°, see below), resulting in 800 occurrences. We also compiled information on documented absences from sampled localities in South America (n = 170), where previous community-level studies of Caprellidae did not detect any specimens of *C. mutica*. While these absences were not used in the SDM, they were used to cross-validate the output of the SDM.

We used 13 oceanographic variables (Table 2) from the BioOracle database v.2.2. (Assis et al. 2018), with a 0.08° (~9.2 km^2) resolution. These variables have commonly been used by previous studies and covered a wide range of biophysical and geochemical conditions in the ocean (Bosch et al. 2018), also reflecting relevant ecophysiological stressors for *C. mutica* as proven by experimental studies (Cook et al. 2007; Lim and Harley 2018). Rasters were masked to include only coastal grid cells, as the species is restricted to shallow waters. The degree of collinearity of environmental predictors was examined by using a variance inflation factor (VIF) analysis where values of VIF > 10 have traditionally been used to claim high collinearity. VIF analyses were carried out using the library 'usdm' (Naimi et al. 2014) in R (ver. 4.1.0; R Core Team 2024). Two variables (mean and range of phytoplankton concentration) showed a high degree of collinearity and were removed from further analyses.

The SDM was built using recommended methodological protocols (Bosch et al. 2018; Feng et al. 2019; Zurell et al. 2020). We created 10,000 random pseudo-absences obtained from all coastal grid cells. We used a Maxent modeling approach, a

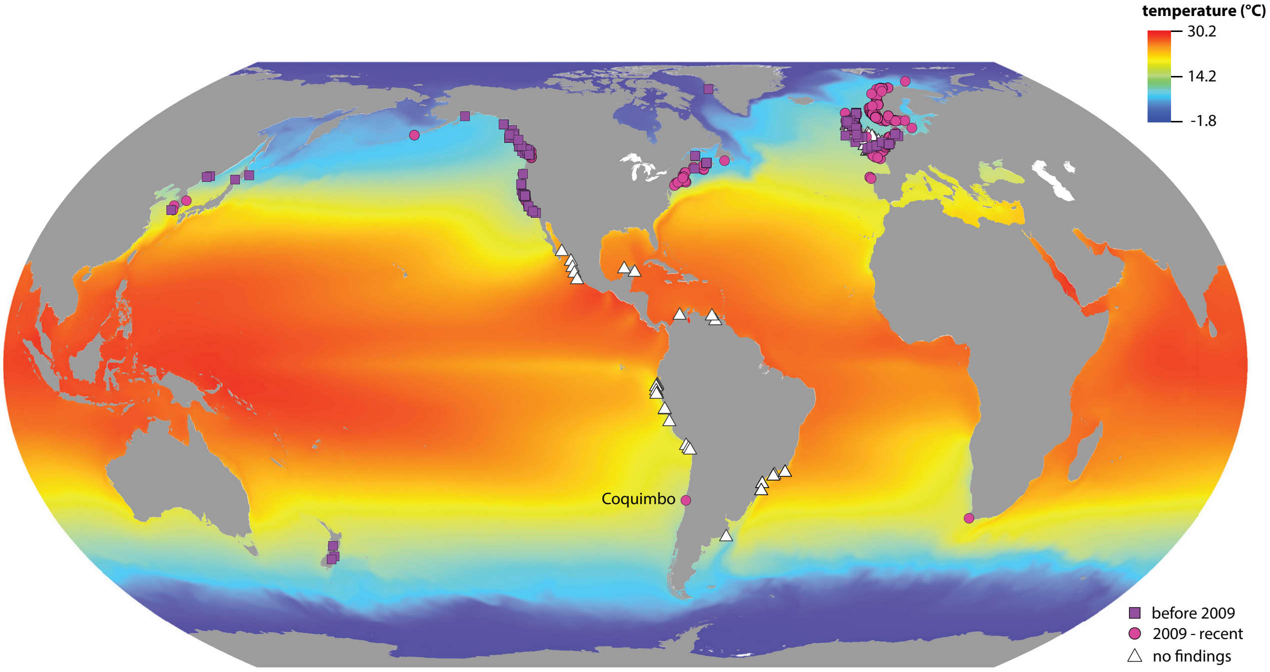

Figure 1. World oceans with mean sea surface temperature (SST) and confirmed reported presences and absences of *Caprella mutica* before and after the year 2009 (i.e. the survey of Boos et al. 2011) considered for this study. 'No findings' refers to sites where previous surveys had examined the caprellid fauna (see also Table 1) without finding *Caprella mutica*.

robust machine-learning algorithm successfully applied to implement SDMs (Elith et al. 2011; Phillips et al. 2017). The model fit was evaluated using the Area Under the Curve (AUC) of the Receiver Operating Characteristic Curve, where values close to 1 indicate a perfect fit. Analyses were conducted using the library 'SDMtune' (Vignali et al. 2020). The model's accuracy was maximized by hyper-parameter tuning and different combinations of the regularization parameter and feature classes. We used a genetic algorithm to assess 150 possible combinations of parameters, evaluating 15 populations in two generations. Genetic algorithms are computational optimization techniques inspired by the process of natural selection (Goldberg and Holland 1988; Alhijawi and Awajan 2024), enhancing model performance, selecting relevant variables, or optimizing parameters when predicting species distributions based on environmental data (Vignali et al. 2020). To ensure the robust spatial transferability of SDMs, we used a four-fold spatial cross-validation scheme based on a checkerboard pattern, implemented in the library ENMeval (Kass et al. 2021) in R. We evaluated the importance of all oceanographic variables in terms of percent contribution and permutation importance and estimated the functional relationship between the occupancy probability and the top predictors using partial dependence plots to isolate the effect of each predictor. A Multivariate Environmental Similarity Surfaces (MESS) analysis was carried out to evaluate areas with non-analog oceanographic conditions. MESS analyses were carried out using the library 'predicts' (Hijmans 2024) in R. Finally, we projected the probability of species occurrence onto the global coasts using ArcGIS Pro (ver. 3.3.0; ESRI Inc.).

Results

Caprella mutica in Coquimbo, Chile and its morphological distinction

In total, seven individuals of *C. mutica* (6 adult males and 1 ovigerous female) were found on August 30th, 31st and September 9th 2022. Besides this newly recorded NIS for this area, the 4 caprellid species *Caprella equilibra*, *Caprella verrucosa* Boeck, 1871, *Caprella scaura* and *Deutella venenosa* as well as the ischyrocerids *Jassa marmorata* Holmes, 1905, *Jassa slatteryi* Conlan, 1990 and *Ericthonius* cf. *rubricornis* (Stimpson, 1853), the maerid *Elasmopus rapax* A. Costa, 1853 (*sensu* Hughes and Lowry 2010), the aorid *Aora typica* Krøyer, 1845, the dexaminid *Paradexamine* cf. *pacifica* (Thomson, 1879) and a stenothoid *Stenothoe* sp. were found coexisting in the amphipod fouling communities of Bahía La Herradura.

In the course of the Marine Biology program of the Marine Science Faculty in Coquimbo, the dominant species in the samples varied between the years, but the most common species were *Caprella equilibra*, *C. scaura* and *C. verrucosa*, and on rare occasions *Deutella venenosa*; the species identified by the students (using Guerra-García and Thiel 2001) were frequently verified by one of the authors (MT). Prior to 2023, no *Caprella mutica* were found, but on 23 June 2023 a few caprellid amphipods examined by the students did not match any of the species reported in Guerra García and Thiel (2001). After closer examination, these individuals were confirmed to belong to *C. mutica*. In addition, two weeks later (7 July 2023), several individuals (adult males and females) of *C. mutica* were collected during a workshop on marine invasive species. The collected individuals have been deposited in the Biological Collection of the UCN (SCBUCN-5533 1 female + 1 male adult; SCBUCN-5537 1 female + 4 male adults; SCBUCN-5561 5 male adults).

The specimens of *Caprella mutica* collected in Coquimbo could be easily distinguished from its two sympatric congeners *C. verrucosa* and *C. scaura* by the absence of a projection on the head. Further, the individuals of *C. mutica* were characterized by numerous spiny projections on the dorsal surface of the pereonites (pereonites 1–7 in females, 3–7 in males), which distinguished them clearly from co-occurring *Caprella equilibra* (Fig. 2). In addition, hyperadult males exhibited dense setation on pereonites 1 and 2, and on gnathopod 2, leading to a conspicuous 'hairy' appearance, which is unique among the known *Caprella* species of the world (Platvoet et al. 1995 as '*Caprella macho*'; Guerra-García and Thiel 2001; Beermann and Franke 2011; Boos et al. 2011; Daneliya and Laakkonen 2012; Heo et al. 2020).

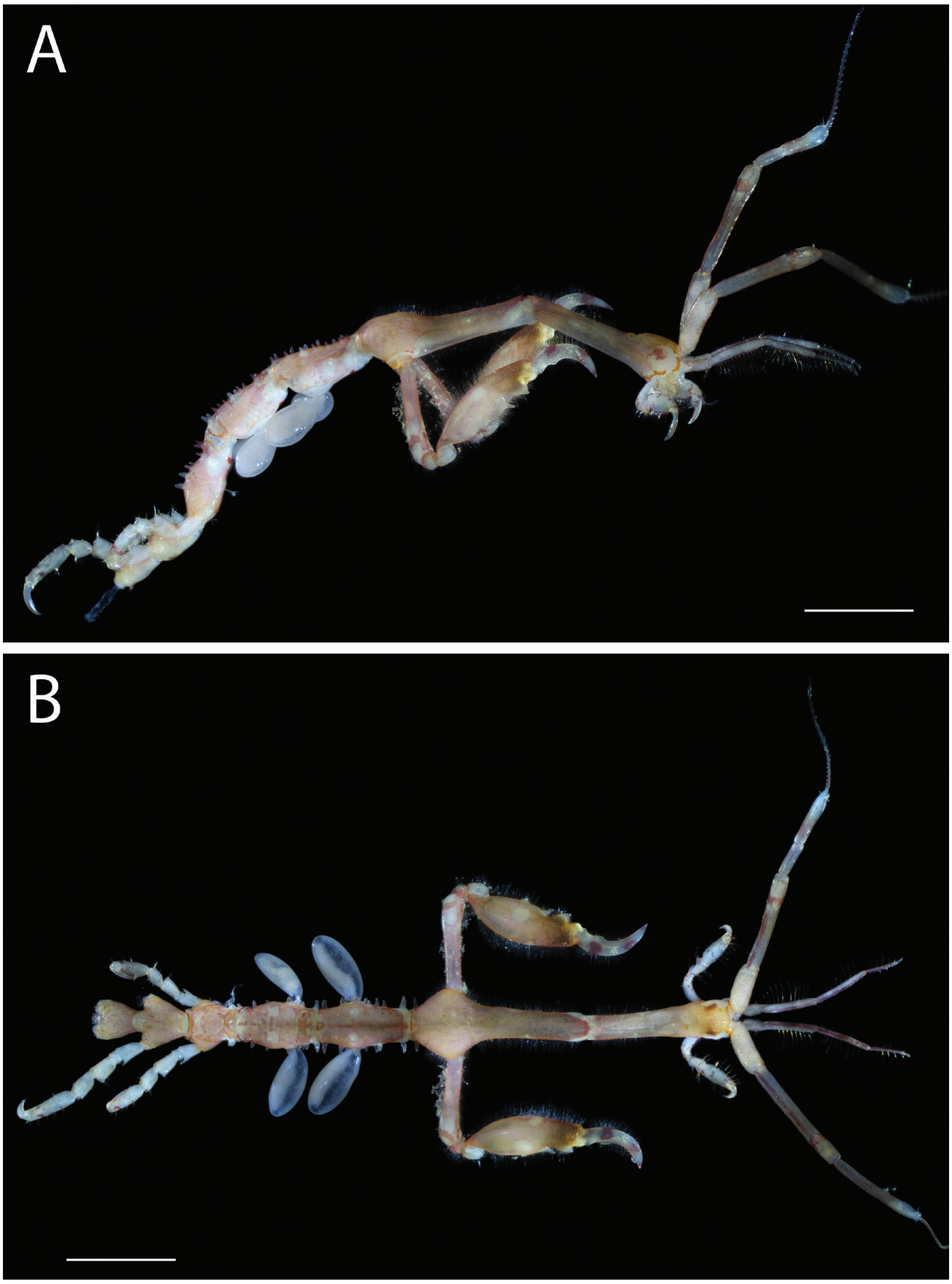

Figure 2. Individual of *Caprella mutica*, collected in Bahía La Herradura (Coquimbo, Chile) on 09 September 2022. Habitus of adult male **A** lateral view **B** dorsal view. Scale bars: 5 mm.

The Asian species *Caprella acanthogaster* Mayer, 1890 shares some morphological characteristics with *C. mutica* that may cause confusion, such as the dorsal spination on the pereonites and the hairy appearance of adult males (Faasse 2005; Daneliya and Laakkonen 2012; Heo et al. 2020). However, the specimens of *C. mutica* found in Bahía La Herradura were characterized by a dense hairy setation all over pereonites 1, 2 and gnathopod 2, whereas the hairy setation in *C. acanthogaster* is restricted to gnathopod 2 only. Further, *C. acanthogaster* bears a pair of two tiny tubercles on the head whereas *C. mutica* specimens from Chile had no tubercles or projections on the head.

Recent caprellid surveys in Central and South America

Over the course of the past 20–30 years, several surveys of the local caprellid fauna had been conducted in several countries of Central and South America (Table 1). These surveys documented a total of 25 caprellid species (of 27 taxa in total) on the Atlantic coast (between 21°N and 38°S), and 16 (of 17 taxa in total) species on the Pacific coast (between 23°N and 30°S). Only four of those species (*Caprella equilibra*, *C. penantis*, *C. scaura* and *Paracaprella pusilla*) were recorded on both Atlantic and Pacific coasts. No findings of *Caprella mutica* were reported in any of these surveys.

Following the initial survey of the local caprellid fauna by Guerra-García and Thiel (2001) and Thiel et al. (2003), the biota growing on aquaculture buoys in the Coquimbo region were again sampled and examined in 2007/08, and all previously identified caprellid species were recorded, but no *C. mutica* was found in that survey (Astudillo et al. 2009).

Species distribution model of *Caprella mutica* and risk of range expansion

The SDM exhibited a high accuracy (AUC = 0.96), and the MESS analyses showed that the model could be extrapolated to ~96% of the coastal grid cells. The model predicted a high probability of occupancy around the native area in Northeastern Asia, and the already invaded areas in Europe and North America (Fig. 3). In general, there was a lower probability of occupancy in the southern hemisphere, except for some areas in South Africa, South Australia, New Zealand, and Chile. Areas with confirmed absences were characterized by low occupancy probabilities (Fig. 3). Along the Chilean coast, the model predicted elevated probabilities (0.30–0.68) of occupancy between 32–42°S, which is 200 to 1,400 km south of the newly confirmed occurrence in the Coquimbo area reported here (Fig. 4). In contrast, the SDM predicted a relatively low occupancy probability (0.07) in Bahía La Herradura.

The mean water temperature (i.e., sea surface temperature) was the top predictor explaining the occupancy of *C. mutica* with a 44% contribution and 74% of the permuted importance (Table 2). The remaining predictors reached much lower contribution and permuted importance, often by one order of magnitude lower and < 10% (Table 2). The partial dependence plot revealed that the effect of the mean temperature was hump-shaped, with maximum occupancy probabilities around 11.3 °C, declining at lower and higher temperatures (Fig. 5).

Table 1. Surveys of the caprellid fauna in Central and South America (references included: ([13] Guerra-García and Thiel 2001; [2] Díaz et al. 2005; [3] Guerra-García et al. 2006; [1] Paz-Ríos et al. 2014; [5] Mauro and Serejo 2015; [8] Sánchez-Moyano et al. 2015; [6] Ros et al. 2016; [9] Alarcón-Ortega et al. 2017; [7] Nunez Velazquez et al. 2017; [4] Cunha et al. 2018; [10] Chunga-Llauce and Pacheco 2021; [12] Tapia-Ugaz et al. 2022; [11] Chunga-Llauce et al. 2023b). Note that only confirmed records on species-level were considered in the table.

Ocean	ATLANTIC										**Sum of Occurrences**	PACIFIC								**Sum of Occurrences**
Country	**Mexico [1]**	**Venezuela [2]**	**Colombia [3]**	**Brazil [4,5,6]**						**Argentina [7]**		**Mexico [8,9]**				**Peru [10,11,12]**			**Chile [13]**	
Latitude	**21°N**	**10°N**	**11°N**	**22°S**	**23°S**	**23°S**	**24°S**	**25°S**	**27°S**	**38°S**	**Atlantic**	**23°N**	**21°N**	**20°N**	**19°N**	**5°S**	**9°S**	**10°S**	**30°S**	**Pacific**
Survey Year	**2013**	**1980–2003**	**1986**	**2012**	**2008**	**2012**	**2009**	**2012**	**2012**	**2015**		**2008**	**2008**	**2002**	**2012–2016**	**2020**	**2019**	**2021**	**2000**	
Aciconula acanthosoma											**0**	x	x		x					**3**
Aciconula tridentata						x					**1**									**0**
Caprella californica											**0**						x			**1**
Caprella danilevskii		x	x		x				x		**3**									**0**
Caprella dilatata				x	x	x		x	x		**5**									**0**
Caprella equilibra		x		x	x	x		x	x	x	**7**		x			x	x	x	x	**5**
Caprella mendax											**0**	x			x					**2**
Caprella penantis		x	x							x	**3**					x		x		**2**
Caprella penantis f. gibbosa											**0**					x		x		**2**
Caprella pitu											**0**	x	x	x	x					**4**
Caprella scaura				x	x	x		x	x		**5**					x	x	x	x	**4**
Caprella suprapiscis											**0**				x					**1**
Caprella verrucosa											**0**						x		x	**2**
Caprellina longicollis											**0**								x	**1**
Deutella caribensis			x								**1**									**0**
Deutella incerta	x	x	x				x				**4**									**0**
Deutella margaritae	x	x									**2**									**0**
Deutella mayeri			x								**1**									**0**
Deutella venenosa											**0**							x	x	**2**
Hemiaegina minuta	x	x	x								**3**									**0**
Hemiproto wigleyi	x	x									**2**									**0**
Liropus guerragarciai							x				**1**									**0**
Liropus isabelensis											**0**	x	x		x					**3**
Mayerella redunca	x	x									**2**									**0**
Mayerella sittropiae							x				**1**									**0**
Metaprotella hummelincki	x										**1**									**0**
Paracaprella carballoi											**0**		x		x					**2**
Paracaprella digitimanus		x									**1**									**0**
Paracaprella dubiaski								x	x		**2**									**0**

Ocean	ATLANTIC										**Sum of Occurrences**	PACIFIC								**Sum of Occurrences**
Country	**Mexico [1]**	**Venezuela [2]**	**Colombia [3]**	**Brazil [4,5,6]**						**Argentina [7]**		**Mexico [8,9]**				**Peru [10,11,12]**			**Chile [13]**	
Latitude	**21°N**	**10°N**	**11°N**	**22°S**	**23°S**	**23°S**	**24°S**	**25°S**	**27°S**	**38°S**	**Atlantic**	**23°N**	**21°N**	**20°N**	**19°N**	**5°S**	**9°S**	**10°S**	**30°S**	**Pacific**
Survey Year	**2013**	**1980–2003**	**1986**	**2012**	**2008**	**2012**	**2009**	**2012**	**2012**	**2015**		**2008**	**2008**	**2002**	**2012–2016**	**2020**	**2019**	**2021**	**2000**	
Paracaprella isabelae											**0**	x			x					**2**
Paracaprella pusilla	x	x	x	x		x	x	x	x		**8**				x			x	x	**3**
Paracaprella tenuis	x				x						**2**									**0**
Pseudaeginella biscaynensis	x	x									**2**									**0**
Pseudaeginella colombiensis			x								**1**									**0**
Pseudaeginella montoucheti					x	x			x		**3**									**0**
Phtisica marina	x	x		x							**3**									**0**
Tritella chibcha			x								**1**									**0**
											25 species									**16 species**

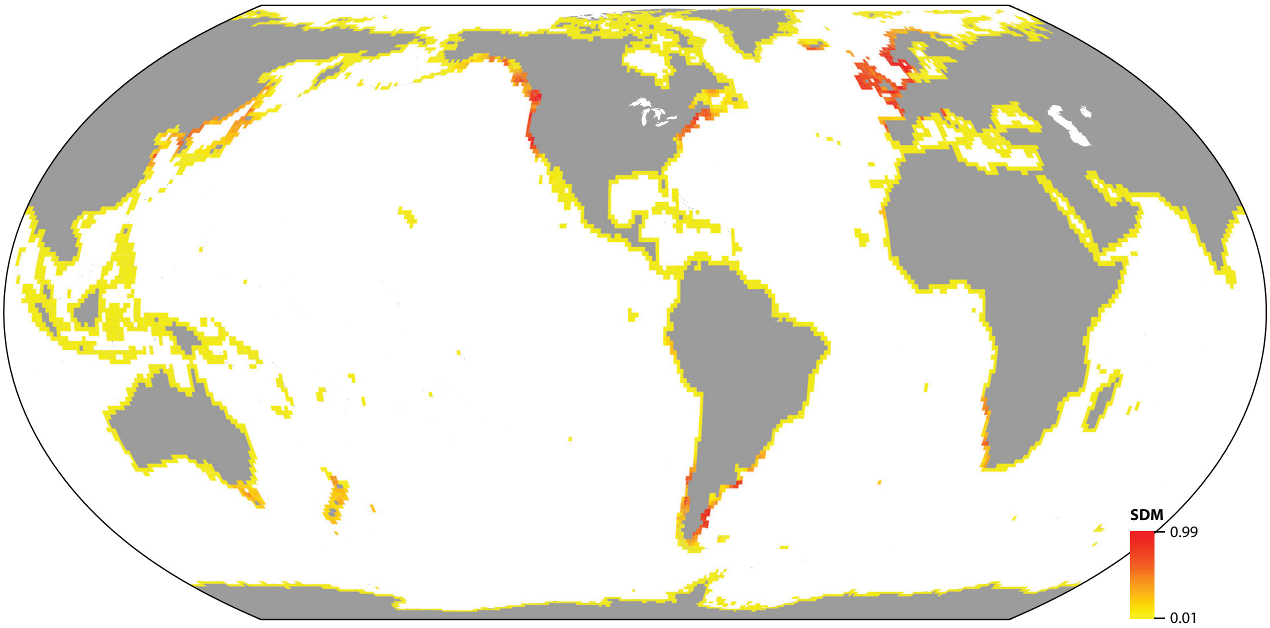

Figure 3. Probability of occupancy of *Caprella mutica* in coastal regions worldwide according to a calibrated SDM. Values closer to 1 (red) indicate higher occupancy probabilities, whereas values close to 0 (yellow) suggest lower occupancy probabilities. The SDM was calibrated at a 0.08° resolution, but is displayed here at a 1° resolution aggregation scale to improve visualization.

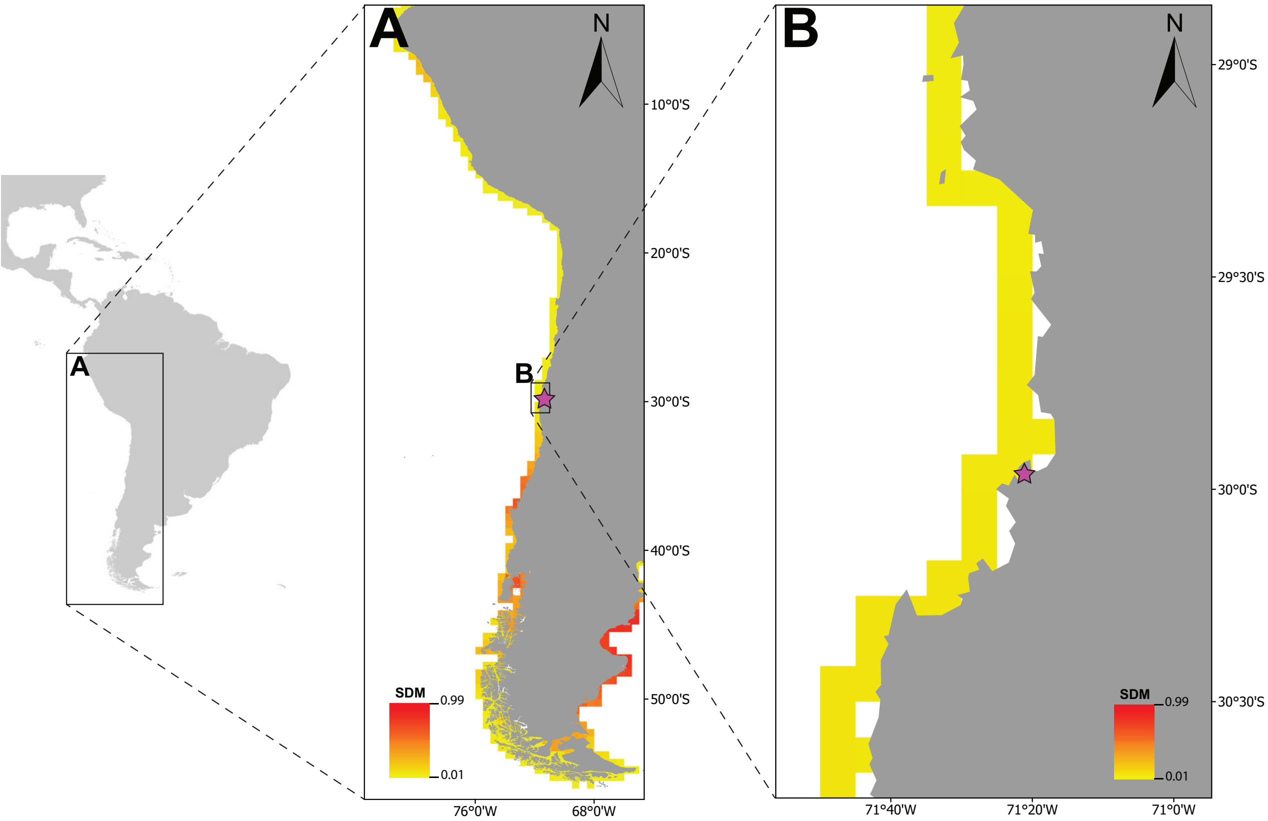

Figure 4. Probability of occupancy of *Caprella mutica* in **A** the Southeastern Pacific and **B** in Coquimbo, Chile according to a calibrated SDM. Values closer to 1 (red) indicate higher occupancy probabilities, whereas values close to 0 (yellow) suggest lower occupancy probabilities. The SDM was calibrated at a 0.08° resolution, but is displayed in **A** at a 0.5° resolution aggregation scale to improve visualization. Asterisks mark the location of Coquimbo in northern central Chile.

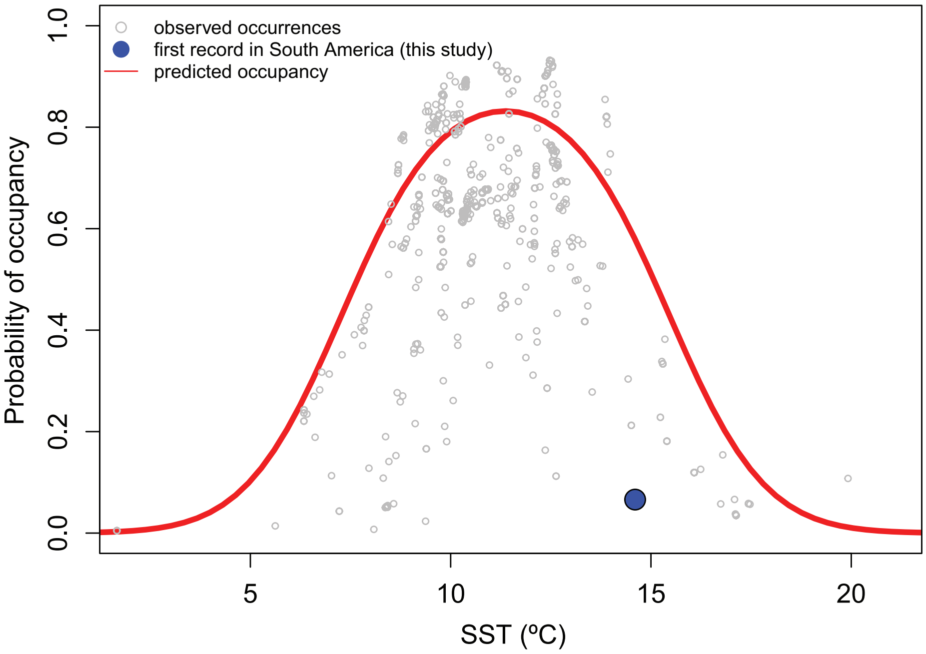

Figure 5. Functional relationships between occupancy probability and mean water temperature according to the species distribution model. Each empty dot represents the occupancy probability estimated for each global georeferenced occurrence. The red line shows the partial dependence plot of the isolated effect of mean water temperature on the occupancy probability. The blue dot indicates the new occurrence site in La Herradura Bay, Chile.

Table 2. Variance inflation factor (VIF) and relative importance of 11 oceanographic variables in an SDM of *C. mutica*. The mean and range of phytoplankton concentration were excluded from the analyses due to their high VIF (> 10).

Variable	VIF	% contribution	Permutation Importance
Temperature mean	4.4	44.3	73.5
Primary productivity mean	2.0	17.7	6.1
Chlorophyll mean	1.6	10.6	1.4
Salinity range	1.4	7.0	2.0
Temperature range	7.5	7.0	3.7
Salinity mean	7.3	5.7	2.6
Diffuse attenuation mean	10.0	2.7	3.2
Chlorophyll range	1.6	2.6	3.0
Silicate mean	8.0	1.3	2.0
Silicate range	3.0	0.7	0.9
Primary productivity range	3.0	0.4	1.5

Discussion

First record of *Caprella mutica* in South America

The current finding of *C. mutica* in Chile represents the first record of this caprellid in South America. Native to the north-east Pacific and introduced to the coasts of North America, Europe, New Zealand and South Africa, *C. mutica* seems to prefer cold-temperate waters (e.g. Arimoto 1976; Ashton et al. 2008b; Willis et al. 2009; Peters and Robinson 2017). Based on the known temperature tolerances of *C. mutica* and given its invasion history, Boos et al. (2011) predicted the species' potential to extend its range to southern Pacific and Atlantic coasts of South America which is now corroborated by our recent finding.

The finding of several adult males and an ovigerous female in Coquimbo in 2022, and the collection of additional adult individuals in 2023 suggests the successful establishment of a population in Bahía La Herradura. However, the observed abundances were quite low compared to the known mass occurrences of *C. mutica* in other introduced ranges (e.g. Buschbaum and Gutow 2005; Peters and Robinson 2017). This could be due to (a) competition with other well-established local caprellid species along with (b) suboptimal environmental conditions for *C. mutica*, or (c) simply be the result of a very recent arrival of this invader in the region. Since *C. mutica* had previously never been observed in Coquimbo despite annual scans of the local caprellid fauna, it is indeed likely that this species has arrived relatively recently. Furthermore, the fact that *C. mutica* has not been reported from other regions in Central and South America, where extensive surveys of the caprellid fauna had been conducted by experts (see Table 1 and references therein), also suggests that this species has only recently arrived in South America. Most of these other studies have surveyed several sites within a country or ecoregion, and explicitly focused on the caprellid fauna (e.g. Díaz et al. 2005; Guerra-García et al. 2006; Paz-Ríos et al. 2014; Chunga-Llauce et al. 2023b), and thus the absence of the highly characteristic *C. mutica* in these surveys strongly suggests that it had not been present in those previous surveys. Since many of these surveys included taxonomical experts for the crustacean family Caprellidae who examined hundreds of specimens, it is considered very unlikely that *C. mutica* would have been overlooked. The population development of *C. mutica* in northern-central Chile must thus be monitored carefully, also with regards to any negative impacts on the local fauna such as the endemic *Deutella venenosa*.

Realized niche space of *Caprella mutica*

Overall, the predicted global occupancy probabilities reflected well the known native range of *C. mutica* as well as its occurrence in areas where it has been introduced (i.e. northern Europe and North America). The modelled predictions of our quantitative approach presented here are roughly in accordance with the "potential range" of *C. mutica* depicted by Boos et al. (2011). In direct comparison to the predicted probabilities in the northern hemisphere, the southern hemisphere seems to be less favorable for this caprellid species. The highest occupancy probabilities for *C. mutica* along the southeastern Pacific coast were observed around 32° and 42°S and were comparatively low in other areas such as Ecuador, Peru, as well as northern and southern Chile. Surprisingly, the SDM predicted only low occupancy probabilities for the Coquimbo Bay (0.07), well below other areas with a similar temperature, which is seemingly in contrast to the recent finding reported here. This new population might thus be living under near-suboptimal conditions that may prevent excessive population growth. A possible explanation could be that the original point of introduction of *C. mutica* to South America may have been located in central-south Chile with its large ports (i.e., San Antonio, Valparaíso and San Vicente) at 33° and 36°S, respectively, where predicted occupancy probabilities increased to up to 0.65. The species may, therefore, already have built undetected populations elsewhere that remain to be found. Further, the local population of *C. mutica* in Bahía La Herradura may be at its physiological limit, reducing the probability of a northward expansion towards northern Chile and Peru. Nonetheless, also if the original point of introduction was indeed in Bahía La Herradura, a further southward expansion to areas where oceanographic conditions could be more favorable, seems likely.

Mean water temperature was the most important variable driving the environmental niche of *C. mutica*. The predicted thermal tolerance according to the SDM, however, is much lower compared to estimations based on ecophysiological experiments (Ashton et al. 2007; Hosono 2011). The median lethal temperature for adults was estimated at 28.3 ± 0.4 °C, while no mortalities occurred at 2 °C, even surviving below-zero temperatures (Ashton et al. 2007). Nevertheless, occupancy probabilities fell below 0.05 at temperatures lower than 5 °C or above 20 °C (Fig. 5). Interestingly, rearing experiments revealed that early stages of C. mutica reach maturity in the range of 10–20 °C, but not at 5 °C (Hosono 2011). All things considered, this suggests that the geographic spread of the species is not only driven by water temperature and it may be strongly co-dependent on the life stage of the animals.

Transport vectors and invasion scenarios along the southeast Pacific coast

The high densities of caprellid amphipods on aquaculture installations and especially on buoys indicate that aquaculture activities might contribute to the dispersal of caprellids along the Chilean and also the Peruvian coast (Thiel et al. 2003; Chunga-Llauce et al. 2023b). In fact, these buoys frequently become detached and are often found floating in coastal waters (Astudillo et al. 2009). The fouling assemblages previously identified on these lost aquaculture buoys contained all caprellid species currently known for the coasts of the SE Pacific (Astudillo et al. 2009). Now *C. mutica* is also found on these highly buoyant substrata, which likely will facilitate its future establishment and spread.

High densities and species richness of caprellids were also found on boat hulls in Peru (Chunga-Llauce et al. 2023b), indicating that small boats also might contribute to the transport of caprellids and other species along the SE Pacific coast. The recent finding of *Deutella venenosa*, a species that previously had only been reported from Coquimbo (30°S) in Chile, from aquaculture structures and boat hulls in Peru (Chunga-Llauce et al. 2023a), indicates that these substrata contribute to the dispersal of caprellids. Rafting dispersal on detached aquaculture structures is also supported by another recent finding of *D. venenosa* on a rope stranded at Ritoque Beach at 33°S (Rech et al. 2023), which could also be expected for *C. mutica* in the future.

Recent records of *Paracaprella pusilla* from Mexico, Costa Rica and Peru (Alarcón-Ortega et al. 2015; Alfaro-Montoya and Ramírez Alvarado 2018; Chunga-Llauce et al. 2022), which had earlier been confirmed at multiple sites near the Pacific entrance of the Panama canal (Ros et al. 2014), suggest another ongoing caprellid expansion along the East Pacific coasts. While most of these findings were made on suspended aquaculture structures, all authors consider transport in/on ships as a more likely cause for the recent appearance of *P. pusilla*.

Several other NIS have recently been reported along the Chilean coasts, including the sea anemones *Diadumene lineata* (Verrill, 1869) (Häussermann et al. 2015), *Metridium senile* (Linnaeus, 1761) (Molinet et al. 2023), and the tunicate *Asterocar-*

pa humilis (Heller, 1878) (Pinochet et al. 2017). In many of these cases, dispersal on ship hulls is considered most likely (Pinochet et al. 2023). For several seaweeds, aquaculture activities and intentional introductions are considered likely causes for recent introductions or range expansions along the Chilean coast (Camus et al. 2022; Jofré Madariaga et al. 2023). Many NIS thrive on floating structures (including aquaculture floats and ship hulls), which facilitates their dispersal and establishment in harbors (Leclerc et al. 2020). All this suggests that shipping activity might have led to the initial introduction of *C. mutica* to the coast of Coquimbo, and that abundant floating structures have then allowed the establishment of a local population.

Conclusions and outlook

The recent finding of *C. mutica* confirms the projection of Boos et al. (2011) who denoted some areas around the world, with South America among them, to be potentially sensitive for the arrival of this caprellid. The global spread of *C. mutica* seems to be ongoing, which is corroborated by recent records from previously unaffected global regions such as South Africa (Peters and Robinson 2017). As the large-scale oceanographic conditions appear suitable for *C. mutica* especially along the South American Pacific coasts and given the vectors and invasion history of other caprellid NIS, a further spread of this invader in South America seems to be inevitable. Therefore, *C. mutica* could now be considered to have become a true 'neocosmopolitan' (*sensu* Darling and Carlton 2018).

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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: JB, MT. Data curation: JB. Formal analysis: MMR, JB. Funding acquisition: MT. Investigation: JB. Resources: MT. Visualization: JB. Writing - original draft: MT, JB, MMR. Writing - review and editing: JB, MMR, MT.

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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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Research Article

A method to quantify jump dispersal of invasive species from occurrence data: the case of the spotted lanternfly, *Lycorma delicatula*

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Abstract

The accuracy of predicting the spread of biological invasions is improved if models explicitly incorporate the two main dispersal mechanisms: diffusive spread and jump dispersal. However, quantitative methods for differentiating these two mechanisms in spatial occurrence data are lacking. We present jumpID, an R package using directional analysis of occurrence data to distinguish between jump dispersal and diffusive spread in biological invasions. We applied this method to occurrence data from the spotted lanternfly (*Lycorma delicatula*) invasion in the US, a pest rapidly expanding its range and impacting the forest and grape industries. Application of jumpID to a dataset of 123,542 occurrence records of spotted lanternfly uncovered 152 dispersal jumps between 2014–2022, with the first jump in 2017, three years after spotted lanternfly's first find. More than half of the dispersal jumps started satellite invasions the year after their detection. The average jump distance did not change over time, with 89% of jumps shorter than 200 km and just three jumps farther than 300 km. The overall spread rate was 41 ± 24 SD km/year, but reduced to 25 ± 11 SD km/year when considering diffusive spread only. Estimating jump dispersal enhances our understanding of species' dispersal mechanisms, provides more robust estimates of diffusion rates for spread models, and helps determine the perimeters for containment and control measures. The R package jumpID is openly available to facilitate invasive spread analysis. jumpID equips scientists and managers with a tool to separate the spread of invasive species into diffusion and jump dispersal components, allowing for more precise parameterization of spread models and directly informing management strategies. Application of jumpID to the spotted lanternfly system indicates that management efforts targeting jump dispersal should focus on a 200-km buffer around the invasion boundary which is where 89% of jumps occur.

Key words: Human-assisted dispersal, invasion boundary, invasion front, invasion spread, invasive species management, long-distance dispersal, secondary diffusion, stratified dispersal

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Introduction

Biological invasions are a costly component of global change and a threat to biodiversity and natural resources (Simberloff et al. 2013; Diagne et al. 2021). Predicting the establishment and spread of invasive populations is crucial to developing control strategies and limiting their impacts (e.g. Barbet-Massin et al. 2018; Verdasca et al. 2021; van Rees et al. 2022). Projections of potential distributions of invasive species have recently started to incorporate dispersal processes to improve the accuracy of the predictions (Václavík and Meentemeyer 2009; Briscoe et al. 2019; Srivastava et al. 2021). In this context, dispersal is often accounted for as a diffusion process through the short-distance, continuous colonization of adjacent locations (Wilson et al. 2009; Lockwood et al. 2013). However, most species expand by stratified dispersal, a combination of short- and long-distance dispersal (Shigesada et al. 1995; Ciosi et al. 2011; Takahashi and Park 2020), and it is widely recognized that rare long-distance dispersal events (LDD) have major consequences for species spread (Trakhtenbrot et al. 2005; Hallatschek and Fisher 2014; Wu et al. 2023). In particular, extra-range LDD result in the establishment of satellite populations at locations away from the invasion range, beyond the limits of the continuous spread, that appear as discontinuous "jumps" in the spatial distribution of an invasive population (Suarez et al. 2001; Nathan 2006; Botella et al. 2022). These dispersal jumps, often due to human-assisted dispersal, become starting points for satellite invasions, a major concern for previously uninvaded areas (e.g. Lanner et al. 2020; Eyer et al. 2021). Yet, the stochastic nature of jump dispersal makes it difficult to identify, predict and incorporate into the projections of potential distributions of invasive species (Nathan et al. 2003; Tobin and Robinet 2022; Wu et al. 2023).

Because of the rarity of LDD, quantifying past dispersal jumps is the best way to understand the distance, frequency, and location at which dispersal jumps occur (Suarez et al. 2001; Nathan et al. 2003; Chivers and Leung 2012). Previously, jump dispersal has been identified with case-specific methods, including visual analyses of distribution data, theoretical models, genetics, or simply expert opinion (e.g. Bossenbroek et al. 2001; Signorile et al. 2016; Eyer et al. 2021; Lee et al. 2022). With increasingly extensive, coordinated survey efforts providing a large amount of occurrence data for invasive species (e.g. Verdasca et al. 2021; Dart et al. 2022; De Bona et al. 2023), there is an opportunity to leverage comprehensive datasets to develop generalized, quantitative methods for identifying dispersal jumps across systems.

Methods using occurrence data for distinguishing diffusive spread from jump dispersal in an ongoing invasion must account for three characteristics of invasions. First, the spread of biological invasions is often anisotropic, meaning diffusion occurs at different rates in different directions from the origin, which leads to invasion ranges with irregular shapes. Dispersal jumps in a direction with slow diffusive spread may be closer to the introduction site than parts of the diffusive range associated with faster spread. Detecting dispersal jumps with occurrence data thus requires delineation of the limits of the diffusive spread in different directions, or else dispersal jumps will be missed in slower progressing sectors. Second, secondary diffusion, i.e. the continuous spread starting from a dispersal jump, constitutes new continuous spread fronts around dispersal jumps (Shigesada et al. 1995; Suarez et al. 2001) that must not be mistaken for new dispersal jumps. Third, methods must account for a temporal component and detect the progression of the continuous spread front over time in both the initial diffusion range and around dispersal jumps to detect jumps only outside of previously colonized areas.

Here, we describe jumpID, a generalized method for distinguishing dispersal jumps from diffusive spread in biological invasions. jumpID identifies dispersal jumps based on spatial occurrence data using a conservative directional analysis to describe the general patterns of jump dispersal and diffusive spread based on occurrence data. jumpID offers a flexible solution for scientists and stakeholders to understand dispersal in invasive populations, facilitating analyses of the features associated with spread patterns, and predictions about areas at high risk of future dispersal jumps.

As a case study, we applied jumpID to spatial and temporal occurrence data for the spotted lanternfly (*Lycorma delicatula*, hereafter SLF) planthopper (De Bona et al. 2023). Native to China and first discovered in the US in Pennsylvania in 2014, SLF is considered an invasive pest because of the threats it presents to the grape and forest industries (Urban and Leach 2023). SLF have established numerous satellite populations despite the enforcement of quarantine zones (see e.g. Pennsylvania Department of Agriculture 2021). Spread models suggest the existence of human-mediated jump dispersal (Ladin et al. 2023) and that SLF can easily spread via jumps to California in the next decade (Jones et al. 2022). SLF is thus an ideal species for demonstrating the utility of jumpID.

Methods

Aim and data requirements

The aim of jumpID is to distinguish dispersal jumps from diffusive spread in biological invasions based on spatial occurrence data. In jumpID, potential jumps are positive occurrences located beyond the limit of diffusive spread for the invasion. jumpID is freely available in R (R Core Team 2023) from an online GitHub repository containing tutorials at https://ieco-lab.github.io/jumpID.

jumpID requires at a minimum a dataset of presences that includes the geographic coordinates at which surveys were conducted. Additionally, datasets including absence data allow systematic verifications of survey effort in presumed uninvaded areas. Datasets with a temporal dimension also allow inquiries about temporal dynamics of spread as well as the identification of jumps enveloped by the progression of diffusive spread over time. If a temporal dimension is included, it is best to split the dataset into time steps that correspond to the temporal scale at which dispersal happens, such as each generation for short-lived species or each year for long-lived species.

Jump identification

Here we provide a brief overview of the jumpID methodology, with detailed descriptions of each component to follow. jumpID detects dispersal jumps by reconstructing the progression of a biological invasion using a directional analysis of occurrence survey data. Starting from a reference point within the invaded range and moving outwards, jumpID identifies spatial discontinuities in the distribution of species' occurrences between the limit of the continuous, diffusive spread and outlying occurrences. All positive records past this discontinuity are potential jumps. This process is repeated for each time step to follow the progression of the continuous spread front. Potential jumps are then confirmed or discarded based on their distance to previous jumps or to secondary diffusion around those. Eventually, the initial occurrence data is divided into three categories: "diffusion", "dispersal jumps", and "secondary diffusion" around dispersal jumps (terms in italics defined in Table 1). A step-by-step tutorial is included in the jumpID GitHub repository.

Table 1. Definitions of frequently used terms in jumpID.

Term	Definition
Types of dispersal	
Diffusion	Continuous colonization of adjacent locations, also called short-distance dispersal or diffusive spread
Jump dispersal	Discontinuous spread of a species' spatial distribution, also called long-distance dispersal
Secondary diffusion	Continuous colonization of locations adjacent to a dispersal event
jumpID terms	
Reference point	Focal point from which all distances for each presence point in the occurrence dataset are calculated.
Distance to the reference point (refDist)	Distance between each presence point and the reference point.
sector	The invaded range is divided into smaller sections (sectors) centered on the reference point and potential jumps are identified within each sector separately.
Minimum jump distance (MJD)	The extent of the spatial discontinuity that makes a presence point a potential jump
Threshold point	The presence point with the greatest refDist that defines the boundary of diffusive spread.
Jump cluster	For temporal jumpID analyses, a group of jumps from the same time step that are all less than the MJD from at least one other jump within the cluster.
Rarefied dataset	Dataset of potential jumps that represents jump clusters as a single point (the jump closest to the centroid of the jump cluster) instead of including all individual jumps.

Data preparation

To start, a "reference point" is chosen for the dataset, and the geographic distance from the reference point to each presence point is calculated ("refDist", Fig. 1A). Ideally, the location of the initial introduction site should be used for the reference point (Renault 2020), or if that is unknown, the center of the invasion core can be used.

To account for anisotropy in species spread, jumpID divides the invaded range into "sectors" centered on the reference point and identify potential jumps within each sector separately (Fig. 1B). Dividing space in any number of sectors will produce accurate results, as the list of potential jumps by sector is then screened through additional pairwise distance calculations across sectors. By default, jumpID divides space into 8 sectors. A demonstration of how computation time may vary with the number of sectors is provided in the jumpID GitHub repository.

Identification of thresholds, jumps, and secondary diffusion

We define the "minimum jump distance "(MJD) as the extent of the spatial discontinuity that makes a presence point a potential jump for a given case study. As outlined by Nathan et al. (2003), the choice of a MJD is inherently case-specific. It must correspond to a high percentage of their dispersal distance that includes all known dispersal mechanisms for outlying points to qualify as dispersal jumps (Trakhtenbrot et al. 2005). We suggest searching the literature for this range of dispersal distances in the invasion of interest, in the species' native range, or in the closest available species. The density of presence data will determine the lowest MJD that can be set in the study system, because the MJD must be higher than the distance separating positive records from continuous spread for jumpID to accurately detect the invasion front. In early invasions with sparse data, the number of sectors may be decreased to provide more data points for the directional analysis. Additionally, the MJD may be adjusted depending on the purpose of the jump analysis. For example, national stakeholders may only be interested in the characteristics of large jumps leading to the colonization of other states or countries (> 100 km), while scientists or local stakeholders may rather be interested in acquiring data on all dispersal jumps to fine tune spread models.

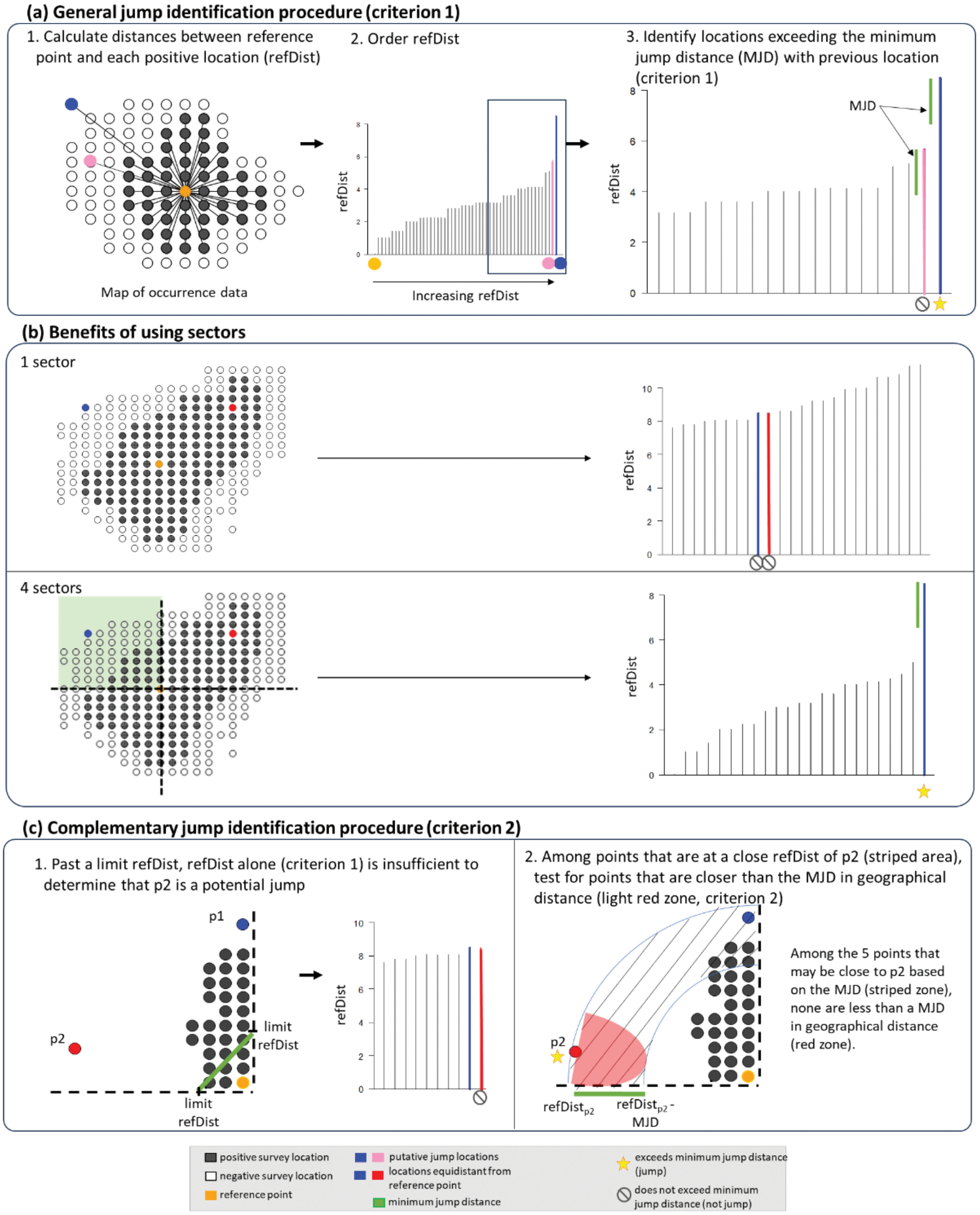

Figure 1. Graphical representation of the workflow used in jumpID **A** representation of the steps leading to jump identifications using the minimum jump distance (criterion 1). The blue point is identified as a jump, while the pink point is not because the difference in distances between the pink point and the preceding point is less than the minimum jump distance (green) **B** graphical demonstration of how a directional analysis accurately identifies dispersal jumps. Since the blue point and red point are equidistant from the reference point (yellow), with only one sector, the blue point is not recognized as a dispersal jump because of its location relative to the shape of the invasion. With 4 sectors, however, the blue point is correctly identified as a dispersal jump using the minimum jump distance (green) **C** graphical demonstration of how criterion 2 identifies potential jumps at longer distances from the reference point. Past a limit "refDist", points may be more distant between them than in "refDist", causing some jumps not to be detected using criterion 1 alone. In this case, a direct, complementary test of the presence of points within an inward MJD of a point (light red zone) determines whether the focal point is a potential jump.

In each sector, presence points are ordered by increasing "refDist" which allows for a directional analysis to detect spatial discontinuities in species presence that mark the limit between diffusive spread and potential jumps (Fig. 1B). Two consecutive presence points (p1, p2) whose difference in "refDist" is greater than the MJD indicate that p2 is a potential jump (criterion 1, Fig. 1A). Because of the gradual increase in sectors' widths over space, past a certain "refDist", two presence points from the same sector may be separated by greater than the MJD from each other, but not their "refDist" (Fig. 1C). Thus, past this limit "refDist", the use of criterion 1 only is insufficient to detect all potential dispersal jumps, and jumpID evaluates a second criterion if criterion 1 does not detect a jump. For criterion 2, jumpID directly verifies that there is no point closer than a MJD in geographic distance inward of p2 among all points with a "refDist" within [refDist_{p2} – MJD; refDist_{p2}]. If criterion 2 is verified, p2 is considered as a potential jump. Criterion 1 filters out most points and limits the longer pairwise distance analysis for criterion 2 to a subset of points that require this complementary test.

If a potential jump is detected according to criteria 1 or 2, the last point before the discontinuity (p1) defines the boundary of diffusive spread ("threshold point") and all remaining points after the discontinuity are listed as potential jumps, starting at p2. Optionally, the user may require the existence of absence points between the "threshold point" and a potential jump to attest that the observed discontinuity is not due to a lack of survey effort.

For datasets with a temporal component, we use the same approach separately for each time step, starting at the earliest time step. For each subsequent time step, we identify potential jumps using the cumulative data from the previous time steps.

Then, from the list of potential jumps from all sectors and all years, potential jumps that are located less than the MJD from any presence point from previous time steps are conservatively eliminated as jumps. In this case, if they are less than the MJD from any "threshold point", they are considered as diffusion; if they are less than the MJD from a previous jump point, they are considered secondary diffusion from this jump.

Overall, the jump analysis thus splits the original presence dataset into three separate datasets: diffusion (core invasion), dispersal jumps, and secondary diffusion from jumps.

Post-treatment of jump clusters

A "jump cluster" is a group of jumps from the same time step that are all less than the MJD from at least one other jump within the cluster. Jump clusters can result from repeated independent jumps to the location in the same time step, from a single jump that led to diffusive spread during the same time step, or even from a jump that diffused for several time steps before the location was surveyed. Because these clusters may not represent independent jumps, jumpID offers the possibility to rarefy the list of dispersal jumps to keep only the jump record that is the closest to the centroid of the jump cluster. The jump dataset obtained after this treatment is called a "rarefied dataset", as opposed to the full dataset that comprises all dispersal jumps, including clustered jumps. This optional rarefication treatment may be insightful depending on the ecological assumptions and the objectives of jump identification.

Case Study: Application of jumpID to SLF

We used a comprehensive open-access dataset summarizing SLF occurrence in the US between 2014 and 2022 at a 1-km^2 resolution (De Bona et al. 2023, data release v2_2023). The dataset comprised 123,542 sampled points, of which 48,601 contained established SLF populations (39%, Table 2, Suppl. material 1: fig. S1). These surveys showed that in just nine years, SLF established in 13 northeastern US states.

Table 2. Summary of jump records identified for each year.

Year	Jump records, annual (rarefied)	Jump records, cumulative (rarefied)
2014	0 (0)	0 (0)
2015	0 (0)	0 (0)
2016	0 (0)	0 (0)
2017	3 (2)	3 (2)
2018	58 (7)	61 (9)
2019	55 (13)	116 (22)
2020	79 (48)	195 (70)
2021	98 (43)	293 (113)
2022	94 (39)	387 (152)

Current evidence indicates that the entire US SLF invasion stems from a single introduction in Berks County, Pennsylvania (Barringer et al. 2015). To date, knowledge on SLF dispersal distances is minimal. Analyses of flight capabilities indicated that adults can only fly short distances (< 40 m) in a single bout (Baker et al. 2019; Myrick and Baker 2019; Wolfin et al. 2019). However, SLF can hitchhike on vehicle exteriors in both the mobile (Elsensohn et al. 2024) and egg (Urban 2020) stages and spread models have found dispersal jumps of more than 150 km in this species (Ladin et al. 2023). For the jumpID analysis, we defined a MJD of 15 km. This scale is likely much larger than their natural dispersal, yet it is small enough to obtain a comprehensive picture of jump dispersal in SLF, including relatively short dispersal jumps that may be caused by a different vector than statewide dispersal jumps. Since SLF complete their full life cycle in one year and have non-overlapping generations (Urban and Leach 2023), we split the dataset into time steps of one year.

We ran jumpID on this dataset with the introduction point as the reference point, a MJD of 15 km, and required checking for negative surveys in discontinuities. We rarefied the jump list with a MJD of 15 km. With the results obtained from jumpID, we calculated jump distances as the shortest distance between a diffusion point and each jump cluster to estimate the minimal distance that SLF had traveled to every jump location.

We conducted several statistical analyses to identify patterns in the jumpID output. We tested whether jump distances increased over the years using a linear model to check if more distant areas were at risk of colonization through jump dispersal over time. To estimate the difference in areas colonized by different dispersal mechanisms, we calculated the yearly invasion radius when considering the two spread patterns together or diffusion only, using the maximal “refDist” in each sector of the original dataset and of the diffusion dataset. To estimate the relative roles of diffusion and jump dispersal in accelerating the species progression, we tested whether the invasion radius increase was slower over time when considering diffusion only compared with all spread together using a linear model. The invasion radius in each sector was the response variable and it was log-transformed to meet assumptions of residuals normality. Year, spread type, and their interaction were the independent variables tested. The yearly spread was calculated as the yearly increase in the invasion radius for diffusion only. Finally, to determine the threat posed by jump dispersal, we determined if jumps were followed by secondary diffusion or rapidly enveloped by the core distribution by calculating if any diffusion point or secondary diffusion point were within the MJD of each jump after one year.

Results

A total of 387 presence records was associated with 152 independent jump events (rarefied jumps, Fig. 2). Clustered jumps were found in 59 of the 152 locations and the remaining 93 locations had only single jumps (Fig. 2). The largest three jump clusters comprised 30 (Winchester, VA, in 2018), 19 (Harrisburg, PA, 2018) and 18 points (Wilkes-Barre/Scranton area, PA, 2019). While jumps were found in all directions around the introduction site, they were disproportionately found west of the invasion range, across Pennsylvania, starting in 2019 (Fig. 2, Suppl. material 1: fig. S2).

We detected the first two dispersal jumps occurring in 2017, three years after SLF was first detected in the US (Figs 2, 3a). The number of new jumps at independent locations then increased every year to reach 48 in 2020, before decreasing to 43 in 2021 and 39 in 2022 (Fig. 3a, Table 2). By comparison, the number of new jumps (including jumps to the same location) rose from 3 to 58 between 2017 and 2018, then overall gradually increased to reach above 90 new yearly jumps in 2021 and 2022 (Fig. 3a, Table 2). The mean number of records per jump was 8.3 in 2018 and 4.2 in 2019, then consistently decreased to 1.6 in 2020, 2.3 in 2021, and 2.4 in 2022.

Jump distances did not increase over time ($p = 0.71$, Fig. 3b). The 152 independent jumps were located on average 87 ± 77 km (SD) from the closest continuous spread front, with 102 jumps (67%) of less than 100 km, 34 jumps (22%) of 100–199 km, 11 jumps (7%) of 200–299 km, 2 jumps (1%) of 300–399 km, and a single jump of over 400 km, in 2021 (Fig. 3c). After one year, 20% of jumps were enveloped by the progression of the diffusion, and among the other jumps, 53% were followed by secondary diffusion, whereas 47% had not spatially expanded (Suppl. material 1: fig. S3).

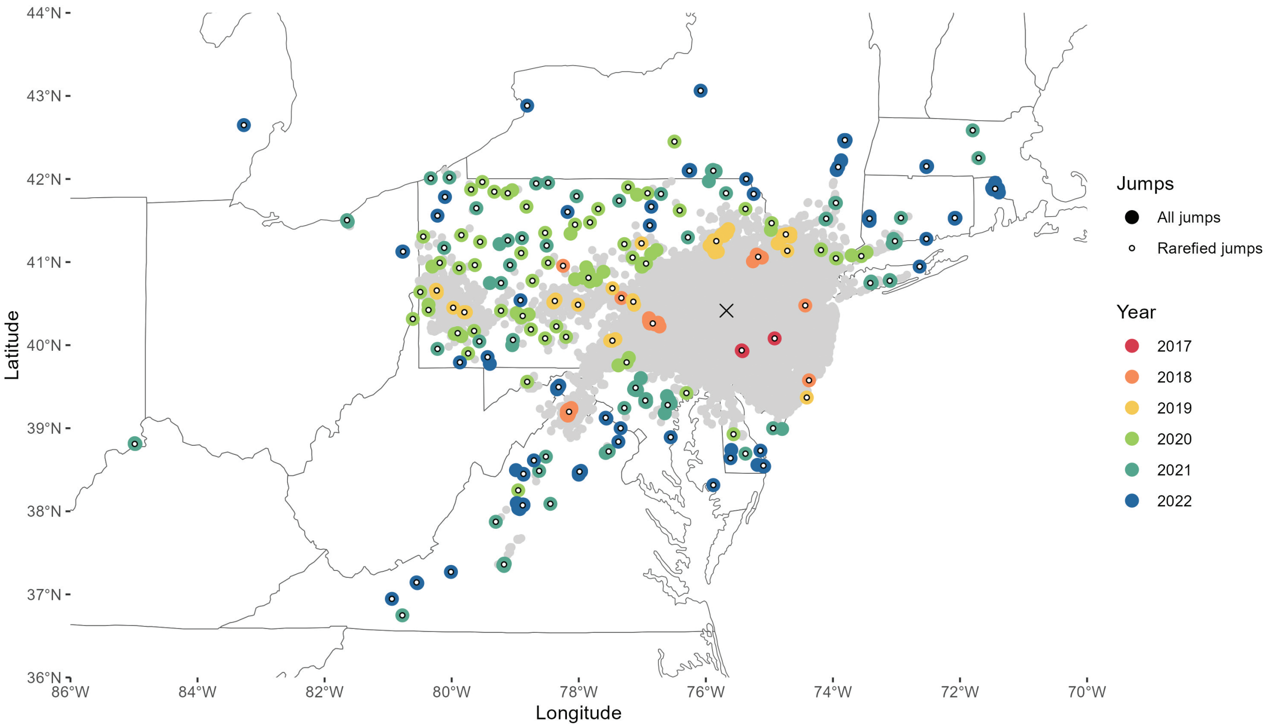

Figure 2. Map of the dispersal jumps identified based on spatial occurrence data of the invasive spotted lanternfly in the northeastern US, colored by year. Gray points represent all established populations recorded up to 2022, including secondary diffusion after jumps. The introduction site in Pennsylvania is signaled by a cross.

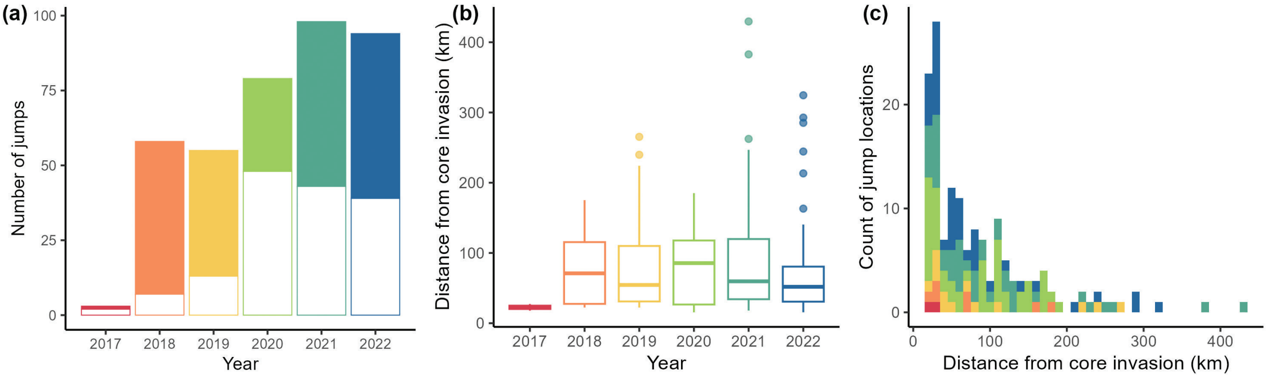

Figure 3. Characteristics of dispersal jumps identified based on spatial occurrence data of the invasive spotted lanternfly in the north-eastern US **a** number of jumps identified per year. The hollow part of bars represents the number of jump clusters (rarefied jumps), the entire bars represent the total number of jumps, including multiple jumps to a single location **b** distribution of the jump distance from the continuous spread front per year, based on rarefied jumps **c** dispersal kernel of jump distance, colored by year.

The invasion radius differed between years (p < 1e-15), spread types (p = 0.005) and their interaction (p = 0.023). In 2022, the invasion radius was on average 334 ± 204 km when including dispersal jumps and 204 ± 89 km when including only the core diffusion (Fig. 4). The radius of the core invasion (diffusive spread) increased on average by 25 ± 11 SD km per year, while the radius of the entire invasion range (diffusive spread, jumps, secondary diffusion) increased on average by 41 ± 24 SD km per year.

Discussion

Accounting for jump dispersal is critical for predicting the spread of invasive species. Jump dispersal allows satellite invasions to occur away from the core invasion, and thus management must enact control at multiple fronts (Suarez et al. 2001; Nathan et al. 2003). Even though jump dispersal is inherently stochastic due to the rarity of long-distance dispersal events that lead to the establishment of a population, risk zones can be identified based on past dispersal jumps. We presented jumpID, a quantitative method to differentiate dispersal jumps from diffusive spread in invasive species based on spatial occurrence data. We documented how this method provides quantitative information on the location, frequency and distance of dispersal jumps through the example of the spotted lanternfly (SLF, *Lycorma delicatula*) invasion in the US.

Analysis of dispersal jumps by jumpID

In our application of jumpID to the SLF dataset, 152 dispersal jumps were identified. The first dispersal jumps were detected in 2017, three years after the species was first discovered. SLF initially dispersed by diffusive spread and had established populations in a restricted area in 2014–2015. By 2017–2018, the invasion range had exploded in size due to stratified dispersal, the combination of diffusive spread and jump dispersal, because jump dispersal disproportionately increases the invasion radius (Suarez et al. 2001). Over half of dispersal jumps have been starting points for satellite invasions, highlighting the threat specifically posed by jump dispersal in the SLF invasion.

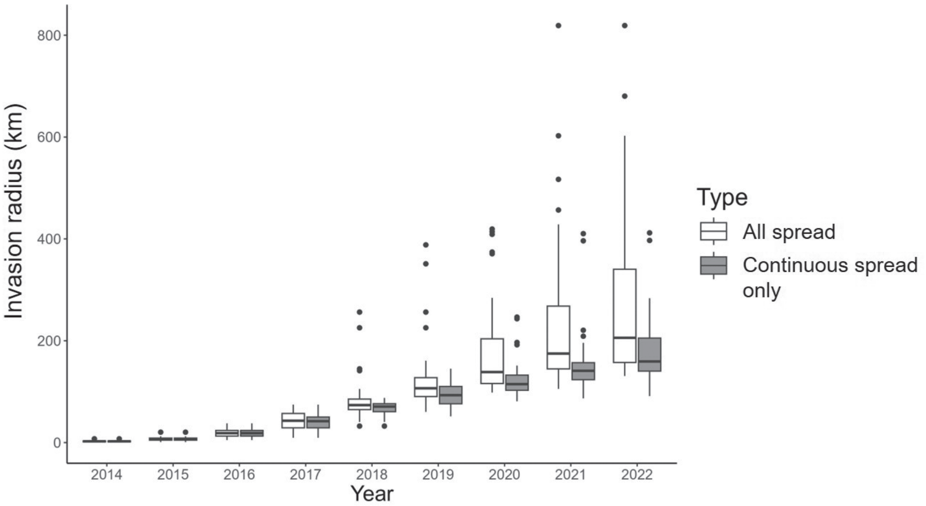

Figure 4. Change in the invasion radius depending on the spread patterns considered. Boxes represent the maximum radius of the invasion per sector for the entire dataset, jumps included (jumps and diffusion), and for diffusion only (diffusion only).

A single jump record was discovered at most jump locations. Such jumps may have included several individuals, or a single gravid female. Indeed, SLF are capable of multiple paternity, and a single mated female can lay multiple clutches of 30–100 eggs (Belouard and Behm 2023). A single gravid female or an egg mass may bring sufficient genetic diversity to sustain the establishment of a population following a dispersal jump. Jump clusters may be caused by a combination of massive propagule pressure (Lockwood et al. 2005) and vectors repeatedly transporting SLF to multiple proximate locations. However, it is most likely that the jump clusters identified were simply the delayed discovery of an original unique jump. In 2018, there was an average of 8 positive records at each jump location, and from 2020, it decreased to 1 or 2 positive records. This indicates that the preventative measures, the monitoring strategy, or both, were effective at decreasing the number of emigrants and/or discovering new infestations earlier, which are both known effective keys to contain biological invasions (Simberloff et al. 2013).

Even though the number of new jumps per year has been above 39 since 2020, it seems to be at least stabilizing in recent years compared to the trends from 2017–2020. Another encouraging signal is that jumps do not occur farther from the continuous spread front over time. It sets a theoretical upper boundary to the area at high risk of dispersal jumps ahead of the continuous spread front. The discovery of these patterns means that the distribution of jump distances may be directly incorporated into spread prediction models. A dual monitoring strategy may be applied ahead of the continuous spread front. First, monitoring may be systematic in a 200-km buffer around the continuous spread front, where most jumps (89%) happen. Second, identifying common characteristics of jump locations would result in a shortlist of at-risk locations to monitor outside the 200-km belt.

It may appear surprising that dispersal jumps were found mostly west of the invasion core. Indeed, jump dispersal is often due to human-assisted dispersal (Nathan et al. 2003) which biases dispersal in preferential axes associated with human activities (Bullock et al. 2018). As such, jumps to major cities along human transportation corridors (e.g., NYC, Baltimore, Washington DC) would have been expected to occur before jumps to central or western Pennsylvania. Therefore, SLF may exploit specific transport infrastructures like freight rail lines that run east to west in this region rather than highways. Landscape analyses of dispersal jumps are a task for future studies to determine more attributes of jump dispersal in SLF based on the list established by jumpID, as well as determining how humans may be facilitating the spread of invaders in general (Bullock et al. 2018; Gippet et al. 2019).

Spread considerations and perspectives on jumpID

By separating dispersal jumps from diffusive spread and secondary diffusion, jumpID also allows a fine analysis of diffusion patterns. Basic estimates of spread rates showed a much slower progression of SLF over time after the jumpID analysis than when diffusion and jump dispersal are considered together. The major benefit of calculating estimates of both diffusive spread and jump dispersal for parameterizing spread models is more realistic spread predictions for invasive species (Robinet et al. 2017; Takahashi and Park 2020).

The choice of a minimal jump distance is a compromise between ecological knowledge, data density, and the purpose of the jump analysis. Indeed, a sparse occurrence dataset may constrain the user to adjust the MJD upwards of the value that would be set using ecological knowledge alone. Setting a MJD larger than the smallest true jump distances would result in unidentified short jumps, i.e. the jump distance kernel being truncated on the left. Intentionally overestimating the MJD may be desirable for users interested in the upper range of jump distances, such as dispersal events that make the species cross state borders. On the opposite, an underestimated MJD would most likely be encountered in systems with limited knowledge on dispersal distances combined with dense occurrence data. It would cause some presence points from the continuous spread to be identified as jumps. An underestimated MJD would be detected in summary statistics and on the maps provided by jumpID: a large proportion of presence data would be identified as jumps, the invasion front would progress very little every year, and most noticeably, jumps would be identified in regions that are visibly within the core invasion (e.g. on yearly maps such as Suppl. material 1: fig. S2). Finally, the MJD is somewhat independent of the continuous spread rate estimates. Diffusive spread may be partly human-assisted, and short-distance vectors accelerate the spread of invasive populations while keeping the invaded range continuous (Lockwood et al. 2013). Here, estimates of yearly spread by diffusion were larger than the minimal jump distance of 15 km considered in this analysis, showing that even short jumps can be identified ahead of highly dynamic invasion fronts. The minimal jump distance is thus not necessarily longer than the average or maximum yearly continuous spread rate.

jumpID is based on occurrence data only, and thus cannot determine the origin of a dispersal jump. For the same reason, jumpID is not intended to detect whether repetitive jumps occurred around the same location over time, as it cannot be differentiated from secondary diffusion around an initial jump. In all cases where a presence point could indicate either diffusive spread or jump dispersal, jumpID makes the conservative assumption that it is not a jump to favor independence in the data.

jumpID optionally accounts for absence data in the distribution of the invasive species as an indicator of survey effort within discontinuities in the species range. When this option is activated, at least one absence point after the continuous spread front is required to validate a dispersal jump. Although it is an imperfect measure of a species' true absence, this use of absence points in jumpID does not alter the results of jumpID as in species distribution models (Václavík and Meentemeyer 2009; Chapman et al. 2019). When absence data are available, this criterion may be strengthened to request a higher number of absence data per gap.

Conclusion

Overall, jumpID is a flexible workflow that can be applied to any species with minimal ecological information to distinguish jump dispersal from diffusive spread based on spatial occurrence data. jumpID is freely available in a public repository and open to modifications to encourage scientists to adapt it to their species of interest. The jump identification and its simple, descriptive analysis is informative for summarizing areas of interest and the overall characteristics of jump dispersal, both temporally and spatially. It opens avenues for more in-depth analyses of jump dispersal and diffusive spread. The insights provided by jumpID results will help stakeholders focus survey efforts on locations predicted at highest risk of future dispersal jumps, thereby increasing the chances of early detection and local eradication before satellite invasions can take place (Simberloff 2014). jumpID thereby contributes to incorporating invasion science into management planning, as largely advocated by the scientific community (Diagne et al. 2020, van Rees et al. 2022).

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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

NB conceived and performed the analysis and wrote the manuscript. JEB, SDB, MRH and IGS contributed critically to the analysis and edited the manuscript. All authors gave final approval for publication. Our study brings together authors from different countries, including scientists based in the country where the data were collected. All authors were engaged early on with the research and study design to ensure that the diverse sets of perspectives they represent were considered from the onset. In addition, local stakeholders were consulted, and their input was incorporated into the study design.

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Data availability

jumpID is available at https://ieco-lab.github.io/jumpID, as well as all data and code used in this manuscript.

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Supplementary material 1

Additional information

Authors: Nadège Belouard, Sebastiano De Bona, Matthew R. Helmus, Isabella G. Smith, Jocelyn E. Behm

Data type: pdf

Explanation note: **fig. S1.** Overview of the data used. **fig. S2.** Results of jumpID, mapped per year. **fig. S3.** Map and occurrences of secondary diffusion per year. **table S1.** Summary of jump identification process per year.

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Review Article

The role of microbiomes in animal invasions: a scoping review

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Abstract

Despite increasing evidence for the contribution of microbiomes to host fitness and evolution, their role in the adaptation and successful establishment of invasive animal species remains underexplored. Animal microbiomes can mediate key host phenotypic traits such as energy metabolism, detoxification and disease resistance. Therefore, harbouring a highly functional microbiome may be advantageous in the context of invasion, where small host populations must rapidly adapt to new environmental conditions. We conducted a scoping review of studies focusing on microbiomes and animal invasions to explore the extent and nature of research efforts on this topic and to identify general patterns that may help elucidate the relationship between host microbial communities and invasiveness. The analysis of 147 articles published between 2006 and 2024 showed a steady increase in the research output on the topic, in parallel with growing interest in biological invasions and technical and theoretical advances in microbiome research. However, the application of new analytical approaches that go beyond taxonomic characterisation remains limited and the research output is still heavily biased towards invasive invertebrates. Although most of the reviewed research was descriptive, a more detailed assessment of a subset of 43 studies using a comparative design revealed some recurring patterns. Host microbiomes in the introduction range tend to diverge from those in the native range, but invasive populations generally retain a core of microorganisms involved in key phenotypic traits such as disease resistance. Studies that have examined the microbiomes of invasive species along their invasion pathway highlight how stochastic events, propagule pressure and population mixing are relevant drivers of microbial community assembly during introductions. Comparisons of the microbiomes of invasive species and co-occurring, outcompeted native species often suggest that some of the observed phenotypic differences driving their interactions are microbiome-mediated. However, to date, only a handful of studies have been able to establish the mechanistic link between microbiomes and host invasiveness using an experimental design. While observational studies remain valuable at this early stage, we advocate for a wider use of novel technologies and experimental approaches to generate robust functional and mechanistic information that will strengthen their inferential value. As more system-specific studies become available, meta-analytical approaches may allow us to uncover broader eco-evolutionary patterns and ultimately elucidate the role of microbiomes in animal invasions.

Key words: Adaptation, alien species, biological invasions, invasiveness, invasive species, metagenomics, microbial community, microbiota

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Introduction

Most animals harbour complex communities of microorganisms, the animal-associated microbiota (Berg et al. 2020) residing in and on their bodies. The microbiota of a single host can include hundreds of different taxa belonging to Bacteria, Archaea, other eukaryotes and viruses. Some of these microorganisms benefit from a relatively stable and protected environment while providing their hosts with functions and services that are often crucial to their fitness (Moran et al. 2019), such as fermentative digestion in ruminants (Mackie 2002; Moraïs and Mizrahi 2019) or skin toxicity in newts (Vaelli et al. 2020).

Over fifteen years ago, Zilber-Rosenberg and Rosenberg (2008) proposed the hologenome theory of evolution, arguing that all eukaryotes should be seen as one with their microbiomes (i.e. the living microbiota plus all of its structural and genetic elements, products and metabolites, Berg et al. (2020)), as they represent a single evolutionary unit (the holobiont) upon which natural selection acts. The need and appropriateness for such a profound shift in paradigm are still debated (Douglas and Werren 2016; Madhusoodanan 2019), but it is generally undisputed that hosts and their microbiota influence each other's ecology and evolutionary trajectories (Koskella and Bergelson 2020; Moeller and Sanders 2020).

In the last decades, evidence for a role of the microbiome in animal fitness, adaptation and evolution has been increasing (Moeller and Sanders 2020), mostly thanks to advances in sequencing technologies that allow for a more in-depth study of complex microbial communities (Larsen and Matocq 2019; Berg et al. 2020). The microbiome appears to be intertwined with a wide range of host traits spanning from nutrition (Dearing and Kohl 2017) to reproduction (Comizzoli et al. 2021), behaviour (Johnson and Foster 2018; Davidson et al. 2020), energy metabolism and thermoregulation (Nicholson et al. 2012; Fontaine and Kohl 2023), immunity and resistance to infections (Gerardo et al. 2020; McLaren and Callahan 2020). Moreover, the microbiota has been hypothesised to increase host resilience to environmental challenges and serve as a mechanism for rapid adaptation, owing to its ability to undergo genomic changes at a much faster rate than animal genomes over short periods of time (Alberdi et al. 2016; Kolodny and Schulenburg 2020; Suzuki and Ley 2020). However, our current knowledge of specific mechanisms and processes linking the microbiome with host adaptation is still largely based on humans and laboratory animals, while mechanistic studies on wild populations are scarce, mostly due to limitations in experimental and analytical approaches (Martin Bideguren et al. 2024).

Biological invasions occur when a — usually small — number of individuals are translocated by humans to a new area outside their natural range, where they establish a viable population and spread away from the point of introduction (Blackburn et al. 2011). Invasions are, therefore, a dynamic process that involves several stages, during which invaders will face multiple biotic and abiotic challenges (Catford et al. 2009; Daly et al. 2023). The severity of these challenges depends on the novelty of the invaded environment and the eco-evolutionary experience of the invading species (Saul et al. 2013; Saul and Jeschke 2015). When the conditions in the new range differ substantially from those in the native range, rapid adaptation may be required for the invasion to be successful (Suarez and Tsutsui 2008; Erfmeier 2013). It stands to reason then that the microbiome, by affecting several key host phenotypic traits, could mediate alien species' adaptation and invasiveness at any of the invasion stages. For instance, having a highly plastic and/or diverse microbial community at introduction could facilitate rapid adaptation to the new environmental conditions and ensure introduced hosts' survival and successful establishment (Alberdi et al. 2016; Kolodny and Schulenburg 2020). At the same time, it is possible that, in some circumstances, it may be advantageous for some alien species to have a highly efficient and specialised native microbiota that is conversely resistant to change. Later on, the microbiome could modulate reproductive traits or disease resistance, promoting proliferation and spread, while possibly giving invaders a competitive advantage over native competitors (McLaren and Callahan 2020; Comizzoli et al. 2021).

However, as yet, the link between microbiomes and animal invasions remains somewhat underexplored. It is important to note that the interaction between microbiomes and invasions is likely to go both ways and disentangling causality can be challenging. As illustrated in Fig. 1 and mentioned above, microbiomes are indeed likely to affect the invasion process by influencing the invasiveness of introduced species, but, at the same time, the invasion process is likely to affect the microbiomes of both invasive hosts and native ecosystems (both the biotic and abiotic components).

For instance, some animal species — especially aquatic or soil invertebrates — have the capability to alter the environmental microbiota surrounding them via microbiome excretion and/or their mechanical or chemical actions. This can increase habitat invasibility and greatly affect local communities, facilitating the establishment and spread of invaders (Malacrinò et al. 2020). This latter mechanism has received considerable attention in plant invasions, where the interaction between soil and rhizosphere microbiomes and invasive plants has long been studied (e.g. Inderjit and van der Putten (2010); Coats and Rumpho (2014); Traveset and Richardson (2014)) and often explored within the framework of the invasional meltdown hypothesis (Simberloff 2006; Zhang et al. 2020). Similarly, the alteration of environmental microbiomes by invasive soil invertebrates has been addressed quite extensively (e.g. Paudel et al. (2016); Ferlian et al. (2018)), while, when considering the host-associated

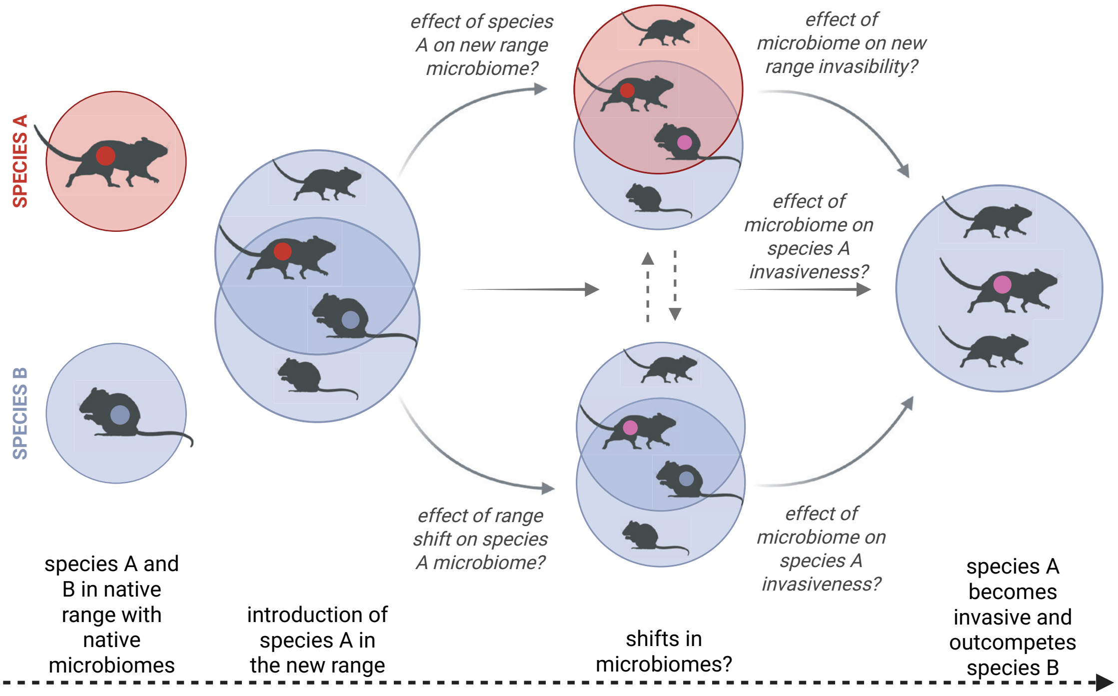


Figure 1. Microbiome dynamics during invasions. Illustration of the potential two-way interactions between animal microbiomes and the invasion process. Created in BioRender (https://BioRender.com/d20m325).

microbial community and how it might facilitate animal invasions, the literature is more sparse. An exception may be insects, a taxon for which several host-microbial symbiotic interactions have been well characterised (Frago et al. 2012; Brinker et al. 2019). Indeed, almost a decade ago, Lu et al. (2016) proposed a conceptual framework of mechanisms by which symbiotic microbes may influence insect invasions.

To collate up-to-date evidence on this topic, extend the analysis to vertebrates and highlight current knowledge gaps and research perspectives, we conducted a scoping review (Arksey and O'Malley 2005; Levac et al. 2010; Peters et al. 2020) of studies dealing with animal invasions and the microbiota. The analysis of 147 articles published between 2006 and 2024, filtered from a pool of 1240 screened publications, enabled us to first explore the extent and nature of the research effort on the topic. Then, we identified general patterns from a subset of 43 studies that focussed specifically on host-associated microbiomes and that, by virtue of their comparative design, may contribute to our understanding of the potential role of animal microbiomes in invasiveness.

Literature search and analysis

The literature search was conducted in May 2024 using both Web of Science and Scopus platforms and following the PRISMA protocol (Page et al. 2021). We searched for the following terms in titles, abstracts and keywords: ("invasive species" OR "alien species" OR "invasive alien species" OR "IAS" OR "invasiveness" OR "non-native") AND ("microbiome" OR "microbiota" OR "microbial communit*" OR "metagenom*" OR "bacterial communit*"). Articles were not limited based on their year of publication, but were only included if they were published in peer-reviewed journals and written in English. The obtained list of articles was first deduplicated, removing 579 records. We then screened the titles and abstracts of the resulting 1240 articles, based on the following set of inclusion criteria:

1. the study included at least one invasive animal as a focal species (hence, studies not pertaining to invasion biology or focussing on plant or bacterial invasions were excluded); and
2. the study investigated the focal species' microbiota and/or the impact of the focal species on the microbiota of either a native counterpart or the local environment; and
3. the study examined the microbiota from a community ecology perspective. Hence, studies that only focussed on the pathobiome or on specific bacteria as a means for biocontrol were excluded.

Concerning the first criterion, although the term "invasive" was sometimes used more loosely in the retrieved studies, we included only studies where the translocation of the focal species to a new geographic range was human-mediated, either intentionally or unintentionally.

Deduplication and title and abstract screening were conducted using Rayyan (Ouzzani et al. 2016). A total of 1076 articles were removed during the title and abstract screening, resulting in 164 articles eligible for the next, full-text, screening. During this second screening, another 17 articles were excluded for not fitting the inclusion criteria listed above. A resulting 147 publications were included in the review. The PRISMA flowchart and the final set of articles are included as Suppl. materials 1, 2, respectively.

Table 1. List of descriptors used to classify the 147 articles included in the present review. A detailed explanation of the study design descriptors can be found in Suppl. material 3.

Article type	Animal group	Animal taxon	Target species	Geographic design	Study design	Microbiota location	Method
Research	Invertebrate	Arthropod	Only invasive sp.	Only native range	Comparative: range and host	Only environment	Amplicon-based sequencing
Review	Vertebrate	Invertebrate (Other)	Only native sp.	Only introduction range	Comparative: range	Gut	Shotgun metagenomics
		Amphibian	Comparative	Only invasion wave	Comparative: host species	Multiple organs/ tissues	Other
		Bird		Comparative	Comparative: IAS[a] traits	Oral	
		Fish			Descriptive: IAS[a]	Skin/Exoskeleton	
		Mammal			Descriptive: impact	Whole specimen	
		Reptile					

[a] Invasive Alien species.

The analysis of included literature was conducted in two stages. First, each of the 147 articles was classified using the descriptors listed in Table 1. Temporal publication trends were explored in terms of target host taxon, study design and methodology for microbiome characterisation. Summary statistics and data visualisation were done using R statistical software (v4.4.1, R Core Team 2023).

Second, we selected the subset of studies that focussed specifically on host-associated microbiomes and used a comparative design to address the hypothesis of a microbial contribution to invasiveness. This led to the identification of 43 articles either comparing: i) the microbiota of an invasive versus a native species, ii) the microbiota of an invasive species in the introduction versus the native range or iii) the microbiota of an invasive species along the invasion wave. From this subset of selected papers, we identified the main recurring patterns and will report a qualitative synthesis of their key findings.

Trends in animal invasions and microbiome research

The 147 papers which met all the inclusion criteria spanned 2006-May 2024, with a median publication year of 2020, demonstrating a steady increase in output over time (Fig. 2a). This increase happened in parallel with the growing interest in biological invasions and with technical and theoretical advances in microbiome research. Original research composed 95.2% (n = 140) of the included papers, while the remaining 4.8% (n = 7) were review articles. Three of the reviews addressed the impact of invasive earthworms on local soil microbiota (McLean et al. 2006; Paudel et al. 2016; Ferlian et al. 2018), three were specific to some invasive insect taxa (*Aedes albopictus*: Garrido et al. (2023); *Spodoptera frugiperda*: Kenis et al. (2023); Tephritidae fruit flies: Hafsi and Delatte (2023)) and the last one is the aforementioned review by Lu et al. (2016) on the role of symbionts in insect invasions.

The classification of the 140 research articles by the methodology used to characterise the microbiota yielded that the vast majority (87.9%, n = 123) of papers applied a targeted amplicon sequencing-based approach (16S rRNA for prokaryotes, 18S rRNA for eukaryotes, ITS for fungi or a combination of these).

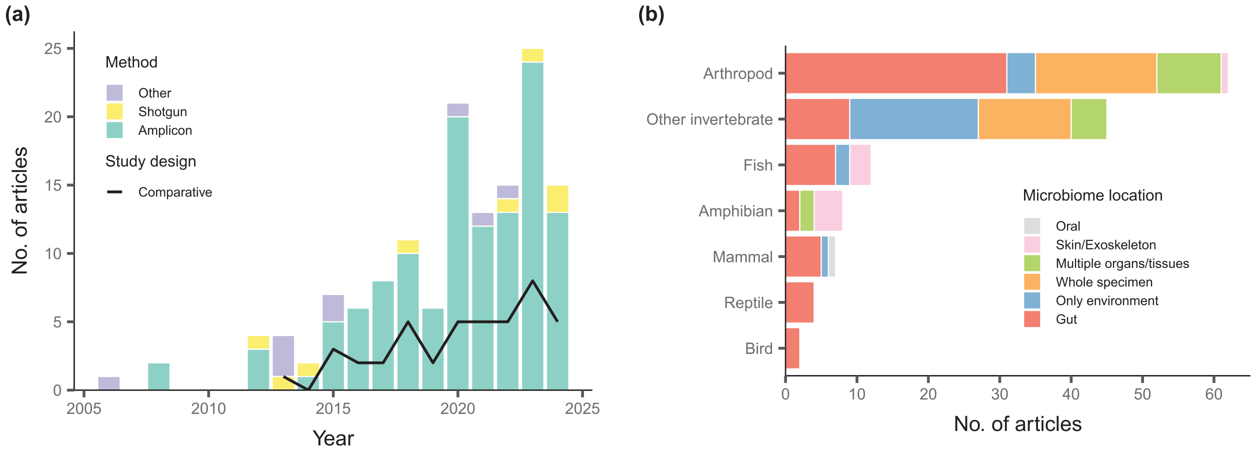


Figure 2. Trends in microbiomes-animal invasions research. Trends in the research output on microbiomes and animal invasions **a** number of articles published by year, methods used to characterise the microbial community and study design (the black line indicates the number of comparative studies); and **b** number of articles published by invasive host taxon and location of the target microbial community.

The remaining 12.1% used shotgun metagenomics (5.7%, n = 8), while the rest relied on other approaches (6.4%, n = 9), such as Restriction Fragment Length Polymorphisms (RFLP), microscopy or staining (Fig. 2a). Amplicon sequencing (or marker-gene analysis) is a well-tested, fast and relatively cheap method to characterise microbial communities from a taxonomic point of view (Knight et al. 2018; Pérez-Cobas et al. 2020). However, it fails to capture functional information that is crucial to link the microbiota structure and composition to host traits (Knight et al. 2018). While indirect functional inference techniques such as PICRUst or Piphilinn (Langille et al. 2013; Iwai et al. 2016) exist, the limited representation and biases of bacterial genome databases challenge the accuracy of indirect functional inferences in wild animals (Pérez-Cobas et al. 2020; Leonard et al. 2025). Shotgun metagenomics on the other hand, by sequencing a broad non-targeted representation of the genetic material present in a sample, enables a direct functional characterisation of the microbiota (Knight et al. 2018; Pérez-Cobas et al. 2020). In particular, genome-resolved metagenomics allows us to assemble the genomes and perform functional annotation even of previously undescribed taxa (Quince et al. 2017), which often represent the bulk of wild animals' microbiomes (Levin et al. 2021; Leonard et al. 2025). However, shotgun metagenomics remains a more costly, time-consuming and data heavy approach, which might explain why its application to invasion biology-microbiome studies is still limited.

Most research articles focused on invasive arthropods (44.3%, n = 62) or other invertebrates (32.1%, n = 44), whereas vertebrates were the focus of 23.6% (n = 33) of the articles (Fig. 2b). Most vertebrate studies regarded fish (n = 12), amphibians (n = 8) or mammals (n = 7), with only a handful of studies on the microbiota of invasive reptiles (n = 4) or birds (n = 2). This strong taxonomic bias in favour of invertebrates is probably due to multiple reasons. Invertebrates and, especially, arthropods, represent the vast majority of animal invasions worldwide (Pyšek et al. 2020; Seebens et al. 2021) and are amongst those with the highest economic impact due to their damage to crops or their role as vectors for human diseases. Although biocontrol falls outside of the scope of the present review, considerable efforts have been directed at identifying specific bacteria that could serve as biocontrol agents against insect pests (e.g. Caragata et al. (2019); Hernández et al. (2024)). Invertebrates are also simpler organisms that pose fewer practical challenges than vertebrates in terms of sampling and experimental manipulation for microbiome studies. In addition, they usually harbour less complex microbial communities that, in some cases, have already been well characterised (e.g. *Wolbachia* spp. in insects, Kaur et al. (2021)). Conversely, some other host taxa are under-represented despite being common invaders. For instance, we found very few studies on the microbiome of invasive birds which could be related to the technical challenges of obtaining high-quality microbial data from their faecal samples compared to other vertebrate classes (Pietroni et al. 2024).

Research also varied in terms of which of the host's microbial communities was studied (Fig. 2b). Most of the research papers focussed on the gut microbiome (42.9%, n = 60), but several studies on invertebrates characterised the microbial community of the entire, homogenised specimen (21.4%, n = 30). Analysing exclusively the skin/exoskeletal microbiome (5.7%, n = 8) was done in one invertebrate study, but was especially common for introduced amphibians and fish, while a single study (0.7%) on mammals focussed exclusively on the oral microbiome. A further 11.4% (n = 16) of studies, all of them on invertebrates or amphibians, jointly analysed microbiomes from multiple tissues or organs within a single invasive animal species. Finally, while analysing some environmental samples along with host samples was common for soil or aquatic invasive organisms, there were several studies (17.9%, n = 25) that did not analyse any host organ or tissue, but focussed exclusively on the microbiome of the surrounding environment to detect any alteration related to the presence of the invasive species.

In terms of design, 39.9% (n = 56) of the research articles were descriptive, with a primary focus on characterising the composition of an invasive species' microbiome or its impact on the surrounding environment, while the remaining 60.1% (n = 84) of research papers included a comparative aspect. Earlier studies were more frequently descriptive in nature, while, from 2018 onwards, there is an increase in the number of more complex, comparative study designs that try to infer the role of the microbiome in the invasive species' adaptation (Fig. 2a).

Patterns in animal invasions and the microbiome

We used the subset of the 43 comparative research papers for a more in-depth analysis to gain further insight into whether the microbiome is a driver or facilitator of invasiveness. The articles included in the subset of comparative studies either compared the microbiome of the invasive species to a native counterpart (17 articles) or the microbiome of the invasive species across its native and introduction range (17 articles) or along the invasion wave (6 articles). Three articles compared the microbiome of the invasive species both against the competing native species and across ranges. Most of these studies still targeted invertebrate hosts (26 articles), but vertebrates were relatively well represented (14 articles). We observed that a few invasive species-microbiome systems have been explored more in depth through multiple comparative studies (listed in Table 2).

The microbiome across geographic ranges

A prominent question when addressing biological invasions and microbiomes is certainly the fate of an invasive host's microbial community after its establishment in a new range. In most cases and across a range of diverse host taxa,

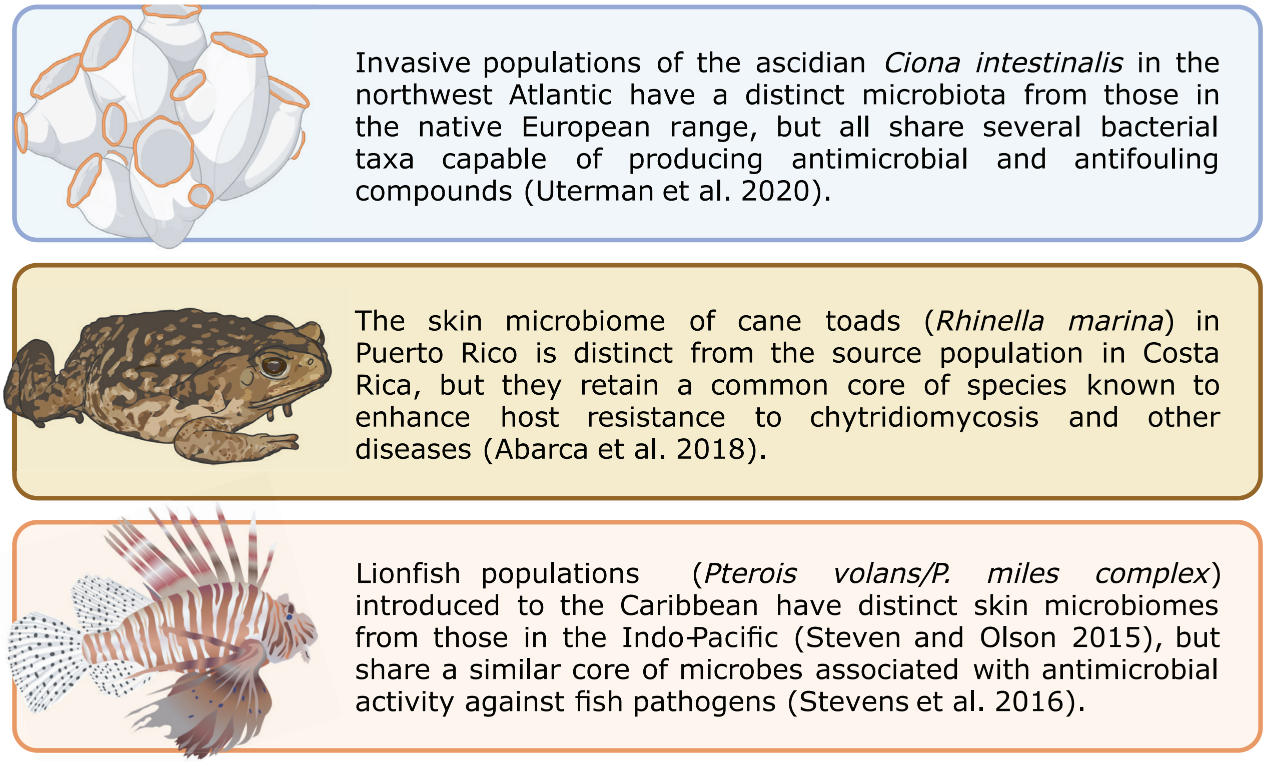
Table 2. List of the invasive animal-microbiome systems assessed by multiple comparative studies with corresponding references.

Invasive host species	References
Asian tiger mosquito (*Aedes albopictus*)	(Minard et al. 2015, Rosso et al. 2018)
Common wasp (*Vespula vulgaris*)	(Lester et al. 2015, Gruber et al. 2019)
Oriental fruit fly (*Bactrocera dorsalis*)	(Liu et al. 2016, Wang et al. 2023)
Warty comb jelly (*Mnemiopsis leidyi*)	(Jaspers et al. 2019, 2020)
Signal crayfish (*Pacifastacus leniusculus*)	(Dragičević et al. 2021, 2024, Grbin et al. 2023)
Lionfish species complex (*Pterois volitans/P. miles*)	(Stevens and Olson 2013, 2015, Stevens et al. 2016)
Red-eared slider (*Trachemys scripta elegans*)	(Qu et al. 2020, Meng et al. 2023)
Cane toad (*Rhinella marina*)	(Abarca et al. 2018, Christian et al. 2018)

microbiomes in the invaded range were found to be significantly distinct from those in the native range (Diptera: Minard et al. (2015); Martinez-Sañudo et al. (2018); Rosso et al. (2018); Hymenoptera: Gruber et al. (2019); Rothman et al. (2021); Tuerlings et al. (2023); ctenophores: Jaspers et al. (2019); ascidians: Utermann et al. (2020); Goddard-Dwyer et al. (2021); fish: Stevens and Olson (2015); Escalas et al. (2022); amphibians: Abarca et al. (2018); Wagener et al. (2022); Leonhardt et al. (2023)). Wild animals' microbiomes are generally highly variable in space and time (Neu et al. 2021; Perlman et al. 2022), but host species are expected to maintain a core of microbes which are most relevant for the host biological function and/or the stability of the community itself (Risely 2020). While there is no unique definition of what constitutes a species' core microbiome (Risely 2020; Neu et al. 2021), the maintenance of a conserved set of microbes (or functions) across native and introduction ranges was observed in several of these systems, notwithstanding the dissimilarity in microbiome structure. Some examples are detailed in Fig. 3 (Stevens and Olson 2015; Stevens et al. 2016; Abarca et al. 2018; Utermann et al. 2020).

There are, however, a few exceptions to this pattern: treehoppers *Stictocephala bisonia* (Szklarzewicz et al. 2020) and brown widow spiders *Latrodectus geometricus* (Mowery 2024) maintain a highly conserved microbiota across their native range and through multiple introduction areas. The microbial communities of these two arthropods are composed of only a handful of vertically transmitted species, strongly suggesting that these are all obligatory symbionts which are crucial for host survival. To a lesser extent, the globally invasive and polyphagous medfly (*Ceratitis capitata*) was also found to harbour very similar communities across multiple biogeographical regions, suggesting that it has attained a highly functional microbial assemblage that allows the host to feed on a wide range of plants (Arias et al. 2022).

Regarding the diversity of microbiomes across ranges, several of the comparative studies found that individuals from invasive populations had, on average, higher microbial richness (Abarca et al. 2018; Utermann et al. 2020; Arias et al. 2022; Escalas et al. 2022) or more complex microbial networks (Gruber et al. 2019) than those from native populations. However, other studies found the opposite pattern (Minard et al. 2015; Lester et al. 2017; Rosso et al. 2018; Goddard-Dwyer et al. 2021) or no difference at all (Lester et al. 2015; Martinez-Sañudo et al. 2018; Rothman et al. 2021; Wagener et al. 2022). No phylogenetic host group appears to be predominantly associated with a particular pattern and, in at least one case, contrasting diversity patterns were even



Invasive populations of the ascidian *Ciona intestinalis* in the northwest Atlantic have a distinct microbiota from those in the native European range, but all share several bacterial taxa capable of producing antimicrobial and antifouling compounds (Uterman et al. 2020).

The skin microbiome of cane toads (*Rhinella marina*) in Puerto Rico is distinct from the source population in Costa Rica, but they retain a common core of species known to enhance host resistance to chytridiomycosis and other diseases (Abarca et al. 2018).

Lionfish populations (*Pterois volans/P. miles complex*) introduced to the Caribbean have distinct skin microbiomes from those in the Indo-Pacific (Steven and Olson 2015), but share a similar core of microbes associated with antimicrobial activity against fish pathogens (Stevens et al. 2016).

Figure 3. Microbiomes across ranges. The microbiome of invasive populations is often distinct from that of populations in the native range, but they typically retain a core of microbial species involved in modulating key phenotypic traits. Created in BioRender (https://BioRender.com/a68e550).

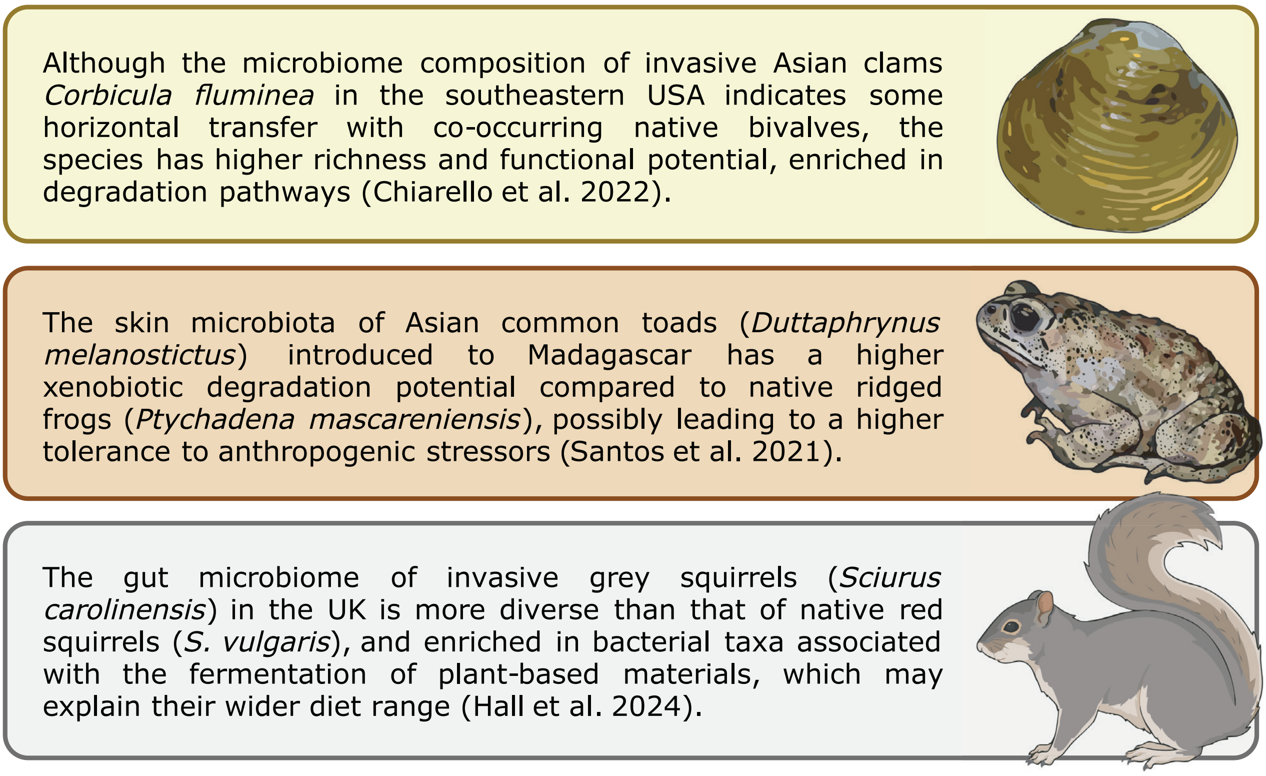
observed by different studies on the same target species (Lester et al. (2015) and Gruber et al. (2019) on common wasps, *Vespula vulgaris*). While it would be tempting to assume that a higher compositional diversity in the microbiome directly translates into higher fitness and adaptability, this is not necessarily the case (Reese and Dunn 2018; Williams et al. 2024). Instead, functional information remains crucial to disclose the relationship between microbiome diversity and host phenotypic traits.

The microbiome across competing species

Some further insight into diversity patterns and invasions comes from those studies that compared the microbiota of an invasive species to that of some ecologically similar, co-occurring native species. In many cases, such studies found that the invader harboured a microbial community that was taxonomically and functionally more diverse compared to the native species (Stevens and Olson (2013) on fish, Duguma et al. (2017) on mosquitoes, Chiarello et al. (2022) on bivalves, Do et al. (2023) on hymenoptera, Hall et al. (2024) on squirrels). However, some other studies found a slightly different pattern, with invaders having a microbiota that was less diverse, but still enriched in some relevant functional traits (Santos et al. (2021) on amphibians, Zuo et al. (2024) on bivalves), showing how diversity metrics alone sometimes might fail to tell the whole story. For instance, Zuo et al. (2024) found that the microbiome of recently established charru mussels (*Mytella strigata*) in China was less diverse than that of native, outcompeted, *Perna viridis* inhabiting the same reef.

Nevertheless, the relative abundance of taxa, metabolites and enzymes related to carbohydrate degradation was significantly higher in *M. strigata*, suggesting that the invader might be more efficient at energy uptake despite harbouring a lower diversity of microbes. Some additional examples of invasive species having a higher microbiome's functional potential than their native competitors are detailed in Fig. 4 and include traits potentially related to a broader diet range (Chiarello et al. 2022; Hall et al. 2024) or to a higher tolerance to anthropogenic stressors (Santos et al. 2021).

Regardless of diversity patterns and similar to what emerged from across-range comparisons, in most cases, the microbiome of the invasive species was clearly distinct from that of co-occurring native species, even when they were phylogenetically very close and/or ecologically very similar (Stevens and Olson 2013; Duguma et al. 2017; Christian et al. 2018; Wilches et al. 2018; Jaspers et al. 2020; Santos et al. 2021; Zhu et al. 2022; Tuerlings et al. 2023; Vasconcelos et al. 2023; Hall et al. 2024). This is hardly surprising, as subtle physiological or anatomical differences between host species can result in relevant differences at the microscale and lead to very distinct microbial assemblages (Maritan et al. 2024), even in species that share the same environment and have largely overlapping niches. Still, a few studies reported some extensive level of homogenisation in the microbiota of invasive and co-occurring native species. This was the case for the exoskeletal microbiota of invasive signal crayfish (*Pacifastacus leniusculus*) and native narrow-clawed crayfish (*Pontastacus leptodactylus*) inhabiting the same river area in Croatia (Grbin et al. 2023), as well as for the gut microbiota of invasive Asian hornet (*Vespa velutina*) and four native, co-occurring *Vespa* spp. in South Korea (Do et al. 2023). Even in these cases, though, some relevant species-specific patterns were identified in the form of differentially abundant taxa.



Although the microbiome composition of invasive Asian clams *Corbicula fluminea* in the southeastern USA indicates some horizontal transfer with co-occurring native bivalves, the species has higher richness and functional potential, enriched in degradation pathways (Chiarello et al. 2022).

The skin microbiota of Asian common toads (*Duttaphrynus melanostictus*) introduced to Madagascar has a higher xenobiotic degradation potential compared to native ridged frogs (*Ptychadena mascareniensis*), possibly leading to a higher tolerance to anthropogenic stressors (Santos et al. 2021).

The gut microbiome of invasive grey squirrels (*Sciurus carolinensis*) in the UK is more diverse than that of native red squirrels (*S. vulgaris*), and enriched in bacterial taxa associated with the fermentation of plant-based materials, which may explain their wider diet range (Hall et al. 2024).

Figure 4. Microbiomes across species. The microbiome of invasive species often has a higher functional potential than that of co-occurring, outcompeted native species. Created in BioRender (https://BioRender.com/v46k439).

Drivers of microbiome assembly during invasions

The microbiome is an assemblage of species acquired by the host through a combination of vertical transfer from its parents and horizontal transfer from other co-occurring organisms and the environment (Maritan et al. 2024). As described in the previous sections, both processes are observed and variably predominant in invasions. At one end of the spectrum, we have invasive species that maintain a completely conserved, vertically transmitted microbiota across ranges (Szklarzewicz et al. 2020; Mowery 2024) and, at the other end, species showing a strongly environmentally-driven assembly resulting in microbial communities that largely overlap with those of co-occurring native species (Do et al. 2023; Grbin et al. 2023). However, in an invasion context, stochastic events related to the invasion process will play a large part in driving the assembly of the "new" microbiome. This would be similar to what often occurs to pathogen communities of invasive species, with rare species lost via founder effects or population bottlenecks and others acquired from the local environment after introduction (Torchin et al. 2003; MacLeod et al. 2010).

Studies that analysed variation in the structure and composition of microbiomes along the invasion path of a species can offer further insight into microbiome dynamics during invasions. For instance, Argentine ants (*Linepithema humile*) were introduced through serial jumps from Argentina to the USA, to Australia and finally to New Zealand and their microbial communities show a progressive decrease in richness and in the relative abundance of core taxa along this path, mirroring a series of bottlenecks in the host population (Lester et al. 2017). Propagule pressure (i.e. a combined measure of the frequency of introductions and the number of individuals per introduction, Lockwood et al. (2005)) is another factor that can potentially affect microbial assemblages in invasive species. Rosso et al. (2018) found that Italian populations of the Asian tiger mosquito (*A. albopictus*) have a depauperate microbial community compared to French populations (Minard et al. 2015) and suggest that this might be the result of fewer introduction events occurring in Italy. Population mixing between invaders of different origin (Wang et al. 2023, see next section) or even hybridisation between different invasive species (Zhu et al. 2021) can further contribute to microbiome differentiation. Asian bigheaded carps (*Hypophthalmichthys nobilis*) and silver carps (*H. molitrix*) introduced into the Mississippi River Basin (USA) were found to extensively hybridise (Lamer et al. 2015). The gut microbiome of hybrids shows several features that are intermediate to those of the parental species, but has also a tendency for higher diversity and presents some unique traits such as a higher relative abundance of taxa involved in ammonium oxidation and an elevated proportion of putative genes related to the digestion of phytoplankton (Zhu et al. 2021).

Decoupling stochastic processes from adaptive shifts in the microbiota can be challenging. Escalas et al. (2022) characterised the gut microbiome of tropical rabbitfish *Siganus rivulatus* and *S. luridus* along their invasion path from the Red Sea to the central Mediterranean and found strong modifications in its structure and composition with increasing distance from the native range. In parallel with a general increase in richness and entropy at the individual level, they also observed a progressive homogenisation of the microbial community both among individuals and between the two species. A reduced variability in community structure within invasive populations can be an indication of stochastic events when coupled to a reduced genetic diversity in the host population, such as in the ascidian

Clavelina oblonga (Goddard-Dwyer et al. 2021). In the case of rabbitfish, however, the genetic structure of Mediterranean populations is similar to Red Sea ones, with no sign of bottlenecks (Hassan et al. 2003; Azzurro et al. 2022). In addition, the rabbitfish microbiota at each site is also distinct from that of the surrounding water and invasive populations have a distinct functional potential related to short chain fatty acids metabolism (Escalas et al. 2022). The observed shift in microbial communities appears then as the result of rabbitfish adapting to a new and more diverse diet in the Mediterranean, where brown macroalgae abundant in the Red Sea are scarce and was likely a key feature for their adaptation to the new range.

As seen with other, non-invasive species (Moeller et al. 2017; Härer and Rennison 2023), a common pattern that emerges from studies comparing different host populations along the invasion wave is that microbiome dissimilarity tends to increase with increasing geographic distance from the source population (Lester et al. 2015, 2017; Jaspers et al. 2019; Escalas et al. 2022; Wagener et al. 2022; Leonhardt et al. 2023). Such differentiation can be extremely rapid: overall the gut microbiome of guttural toads (*Sclerophrys gutturalis*) introduced in Cape Town (South Africa) 20 years ago has diverged compositionally from their source population, but extensive divergence was also found between core and front populations within the introduction area itself, suggesting rapid alteration during range expansion (Wagener et al. 2022). Similarly, along the expansion range of the the oriental fruit fly (*Bactrocera dorsalis*) in China, the microbiome of early-established populations (dating back to the 1970s) is clearly distinct and more diverse than that of late-established ones (2000s) (Liu et al. 2016).

Finally, it must be considered that several vertebrate invasions result from accidental or intentional release from captivity and captivity is another factor that can strongly alter microbial assemblages (McKenzie et al. 2017; Alberdi et al. 2021; Dallas and Warne 2023). As an example, when comparing the gut microbiome of free-ranging and captive American minks (*Mustela vision*) and European minks (*M. lutreola*) in Spain, van Leeuwen et al. (2023) found that captivity led to depauperate communities and its effect even surpassed differences between host species. This reduction in richness was particularly strong in invasive individuals that experienced poorer welfare and management conditions while in captivity (i.e. fur farming vs. captive breeding programme for repopulation).

The microbiome as a driver of invasiveness

Although most of the research included in the present review is observational, some recent studies explored the mechanistic link between microbiome composition and invasiveness traits through experimental set-ups.

Fontaine and Kohl (2020) compared the response of tadpoles' gut microbiota to a temperature gradient in two ecologically similar North American frog species: the globally invasive bullfrog (*Lithobates catesbeianus*) and the congeneric green frog (*L. calmitans*), which is instead restricted to its native range. As predicted, the invasive species showed a highly plastic microbiota that responds more rapidly to temperature changes, both compositionally and functionally, which might contribute to its great invasion success. Specifically, many microbial pathways were altered in bullfrogs in response to treatment and about a third of these were enriched at warmer temperatures, suggesting an adaptive change in microbial metabolism. Conversely, microbial metabolism in green frogs showed fewer and slower changes, and the altered pathways all had a decreasing trend, suggesting metabolic depression.

In a similar experiment, Meng et al. (2023) analysed the response of three freshwater turtles in China (native *Mauremys reevesii*, alien - but not invasive - *Pseudemys nelsoni* and invasive *Trachemys scripta elegans*) to varying concentrations of ammonia in terms of swimming performance, gut microbiota composition and liver metabolomic profiles. In this case, however, only the latter were significantly altered in response to treatment and, in particular, amino acids and energy metabolites were strongly reduced in *M. reevesii* and *P. nelsoni*, but not in *T. s. elegans*, suggesting that the invasive species may be more resistant to pollution. Conversely, the diversity and structure of gut microbial communities were unaffected by ammonia exposure in all species and, although the authors report that the relative abundance of some microbial genera varied with ammonia concentration, the lack of functional information on microbial metabolism does not allow much inference on the possible adaptive significance of such changes.

Another experiment addressing the role of microbiomes in invasions concerns the fall webworm (*Hyphantria cunea*), a globally invasive North American moth. During the last decades, the species has spread throughout China's temperate regions and it is now expanding further south, into subtropical areas. Zhang et al. (2024) characterised the gut microbiome of *H. cunea* larvae across these regions, to explore its influence on survival and adaptation to such a broad range of habitats and host plants. Larvae sampled on different plants showed highly dissimilar microbiomes and, in particular, larvae from southern populations sampled on toxic *Metasequoia glyptostroboides* had the most diverse and distinct microbial community, enriched in taxa associated with detoxification. The authors further showed that larvae from northern populations fed with *M. glyptostroboides* have higher mortality compared to southern ones that are adapting to this new host plant. A final microbiota transplantation experiment from southern larvae into northern larvae boosted the survival rate of the latter on *M. glyptostroboides*, confirming that the microbiome is playing a key role in the rapid adaptation of this invasive species to new habitats and food sources.

As mentioned before, repeated introductions followed by population mixing could promote increased diversity of microbial communities and prove beneficial for host fitness. Populations of the invasive oriental fruit fly (*B. dorsalis*) in northern China appear to be hybrids of lineages originating from different biogeographical regions following multiple introductions (Aketarawong et al. 2007; Qin et al. 2018). In a recent study, Wang et al. (2023) compared the fitness and microbiome of lab-reared lines of *B. dorsalis* originating from outbred, invasive populations with those of lines originating from inbred populations. They found that outbred flies had higher heterozygosity and fitness (i.e. survival rate, pupal weight and egg output), as well as a compositionally and functionally richer microbiome and higher concentrations of several amino acids. Supplementing inbred individuals with some of these amino acids and feeding them with the spent diet from outbred flies both resulted in a significant increase in their fitness. The authors further characterised the intestinal transcriptome of the two phenotypes, showing that several pathways related to immunity and growth are upregulated in outbred individuals. This led them to conclude that compositional changes in the microbiome determine an increase in the concentration of specific amino acids which leads, in turn, to transcriptional shifts promoting growth, development and ultimately invasiveness.

Knowledge gaps, best practices and research priorities

Our review of the existing literature shows that most of the published studies are still descriptive, that technical and analytical methods are far from standardised and that some host taxa are still poorly represented, hindering the possibility of conducting robust quantitative meta-analyses. However, publication trends suggest that interest in the topic is steadily growing and we are confident that more data will soon become available, allowing for meta-analytical approaches that will enable researchers to address broader eco-evolutionary questions regarding the role of animal-associated microbiomes in invasions.

For instance, contrasting diversity patterns emerged from both across ranges and across species comparisons, but the number of studies is still too limited to identify any consistent associations with, for example, host phylogeny. Another interesting question to address would be whether it is more advantageous for an invading species to have a more plastic or conversely a more resistant microbial community. From a slightly different perspective, one could also ask whether some bacterial taxa — or functions — might be more beneficial to conserve — or acquire — than others. It is likely that the answers to such questions would be highly dependent on the host species, the invasion context and the specific functional role of the different microbial taxa, but as more data become available, meta-analyses could potentially reveal broader underlying patterns related to the characteristics of the invaded habitats or the phylogeny and/or niche specialisation of the host or microbial taxa.

Further system-specific research is, therefore, needed to enable researchers to address these broader questions, but it is important that future studies adhere to some common standards in order to be comparable and have inferential value. For instance, our review highlights that the vast majority of comparative studies are still observational in nature, echoing the findings of a recent systematic review on microbe-driven adaptation in wild vertebrates (Martin Bideguren et al. 2024). While we acknowledge that conducting experimental studies in wild animals, especially vertebrates and on a large scale can be challenging, experimental evidence is needed to elucidate the mechanistic link between microbiome composition and host adaptation (Kohl 2017; Davidson et al. 2020; Koh and Bäckhed 2020). In particular, experimental manipulation of hosts' microbiota through faecal transplants or antimicrobial administration is a robust way to test causality in microbiome-related hypotheses (Koh and Bäckhed 2020). For example, thanks to a microbial transplant, Zhang et al. (2024) were able to demonstrate that the adaptation of the invasive fall webworm (*H. cunea*) to new host plants is microbiome-mediated.

Nevertheless, we argue that, at this early stage, comparative, observational studies are still valuable to shed light on whether animal microbiomes may be relevant drivers of invasiveness in any way. In such a high-dimensional and complex system as the host and its microbiome, correlative studies can help to sort out potentially influential patterns that can later be addressed by an experimental approach to prove causality and determine its direction (Davidson et al. 2020). Our review shows, for instance, that comparisons across geographic ranges can reveal changes in microbiome composition and/or functionality potentially linked to the adaptation to the new range and to a successful invasion. Similarly, comparisons between different populations along the invasion path can shed light into processes affecting the assembly of microbial communities. Lastly, comparing the microbiome of invasive species against that of outcompeted, phylogenetically close native species can offer some insight into whether differences in microbiome functionality play a part in their competitive interaction.

However, two requirements are essential for such correlative studies to be meaningful and have some inferential value: first, the use of an appropriate sampling design and second, the generation of robust functional information alongside taxonomic data. Wild animals' microbiomes usually show high intra- and inter-individual variation; hence, working at the appropriate spatial and temporal scales, sampling multiple populations, as well as choosing the right microbial taxonomic resolution are fundamental to avoid sampling artefacts (Knight et al. 2018; Neu et al. 2021; Zoelzer et al. 2021; Degregori et al. 2024). For instance, Tuerlings et al. (2023) found that the microbiome of common wasps (*V. vulgaris*) in the introduction range was completely distinct from the one in the native range, but they point out that this result might be an artefact linked to the limited number of populations sampled in the latter. Similarly, Qu et al. (2020) found that native (*Chinemys reevesii*) and invasive (*T. scripta elegans*) freshwater turtles had similar gut microbiota, but they acknowledge that their small sample size might have failed to capture the full extent of the microbiome composition in the two species.

In terms of functional inference, we advocate for a more widespread use of shotgun metagenomics, since reliable and complete functional information is critical for drawing conclusions about observed microbiome shifts or differentially abundant microbial taxa (Quince et al. 2017; Pérez-Cobas et al. 2020). Whenever cost is a relevant constraint, a possible solution would be to use a mixed approach (e.g. Jang et al. (2022); Zhang et al. (2023, 2024)), applying amplicon sequencing to the entire sample set and shotgun to a representative subset, to derive functional information while limiting sequencing costs. However, the application of different analysis methods should be carried out considering the biological and technical factors that can easily lead to biased or inconclusive results (Aizpurua et al. 2023; Pietroni et al. 2024). Large-scale standardisation initiatives like the Earth Hologenome Initiative (Leonard et al. 2025) can be instrumental in achieving that goal. The ultimate aim should be to couple microbiome compositional and functional information with measures of host fitness (e.g. Wang et al. (2023); Zhang et al. (2024)) and/or other -omics approaches, such as metabolomics (e.g. Utermann et al. (2020); Zuo et al. (2024)) or transcriptomics (e.g. Wang et al. (2023)), to both establish causal relationships and gain insight into the mechanistic processes linking microbiome dynamics with animal invasiveness.

Conclusions

Our review of the existing literature shows that the attention of the scientific community to the role of the microbiome as a potential driver of animal invasions has steadily increased over time, but research is still taxonomically biased and mostly observational in nature. The analysis of the subset of comparative studies shows that, in most systems, the host microbiome undergoes relevant changes during the introduction process and many of these shifts appear to have some adaptive value. Several studies also highlight the importance of stochastic processes in determining the post-invasion microbial community. However, to date, only a handful of experimental studies have demonstrated the mechanistic link between the microbiota and invasiveness in an animal species. More such studies are needed to elucidate whether adaptive shifts in microbial communities following invasion are a common occurrence. We believe that observational studies remain valuable, but only when combined with a robust sampling design and strengthened by measures of host fitness and the adoption of new analytical approaches that allow for more robust functional inference. As more complete, system-specific studies become available, meta-analytic approaches will allow researchers to compare the dynamics of microbial communities across multiple invasive species and ecosystems and, potentially, uncover broader eco-evolutionary patterns related to the role of microbiomes in animal 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

Conceptualisation: CR, AA. Data curation: CR. Formal analysis: EB. Investigation: CR, EB. Methodology: CR, EB. Supervision: CR, AA. Validation: LAW, AA. Visualisation: CR, EB. Writing - original draft: CR. Writing - review and editing: CR, EB, LAW, AA.

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Data availability

No new data were analysed or generated for this study. The full list of articles included in the review is provided as Suppl. material 2.

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Supplementary material 1

PRISMA flowchart

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Data type: pdf

Explanation note: PRISMA flowchart illustrating the systematic review process.

Link: https://doi.org/10.3897/neobiota.98.145939.suppl1

Supplementary material 2

List of reviewed articles

Authors: Claudia Romeo, Elsa Brenner, Lucas A. Wauters, Antton Alberdi

Data type: csv

Explanation note: Full list of the 147 articles included in the review, their metadata and associated descriptors.

Link: https://doi.org/10.3897/neobiota.98.145939.suppl2

Supplementary material 3

Study design descriptors

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Data type: pdf

Explanation note: Description of the categories used to define the study design of the assessed articles.

Link: https://doi.org/10.3897/neobiota.98.145939.suppl3