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Extent, impacts and drivers of oystershell scale invasions in aspen ecosystems

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Abstract

Invasive herbivores that kill foundation tree species pose a major threat to forest ecosystem sustainability. One important foundation tree species in the interior western United States is quaking aspen (Populus tremuloides Michx.), which is threatened by recent outbreaks of an invasive insect, oystershell scale (Lepidosaphes ulmi Linn.; OSS). OSS outbreaks were first reported in 2016, when OSS began causing dieback and mortality of aspen in wildland forest settings in northern Arizona. Since then, OSS has been observed in other locations across Arizona and in other western states, and recent studies in Arizona have highlighted the threat that OSS poses to aspen sustainability, warranting a comprehensive survey of OSS invasions and their impacts on aspen ecosystems. We sampled aspen populations across Arizona and addressed three questions: (1) What is the geographic extent of OSS in Arizona? (2) What impacts does OSS have on aspen? (3) Which biotic and abiotic factors influence the proportion of aspen stems infested by OSS? OSS was present in 29% of our 220 study plots and had a negative impact on aspen forest health. OSS was associated with crown damage and tree mortality, especially of intermediate-sized, recruiting stems. Climate was the most important driver of OSS infestation, with warmer, drier conditions resulting in significantly more OSS. OSS was also associated with less recent fire, presence of ungulate management strategies (e.g. fenced exclosures) and stands with a greater density of aspen saplings. We conclude by providing OSS monitoring and management recommendations, based on our findings, and emphasise that active management such as prescribed fire, reduced reliance on ungulate exclosures or thinning - is required to suppress OSS populations and mitigate damage to aspen ecosystems.

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Introduction

Invasive species pose a major threat to sustainability of forest ecosystems (Chornesky et al. 2005). Of particularly high concern are invasive species that kill foundation tree species, resulting in reductions to biodiversity and other ecosystem services (Ellison et al. 2005; Ellison 2019). Unlike keystone species, which are low in abundance but have an outsized influence on ecosystem dynamics, foundation species are common at the landscape scale and often abundant at smaller spatial scales (Ellison et al. 2005; Ellison 2019). An important foundation tree species in the interior western United States is quaking aspen (*Populus tremuloides* Michx.), which has the widest distribution of any tree species in North America (Little 1971; Perala 1990). Despite its continental abundance, aspen is relatively uncommon along the south-western edge of its range, where aspen occupies less than 2% of forested land (Johnson 1994; Gitlin et al. 2006; Halbritter and Bender 2011; Zegler et al. 2012; Crouch et al. 2023). Additionally, although they occupy a small portion of the landscape, aspen ecosystems have high conservation value because they make disproportionately large contributions to biodiversity and other ecosystem services compared to more abundant conifer-dominated stands (DeByle 1985; Chong et al. 2001; LaMalfa and Ryle 2008; Kuhn et al. 2011; Rogers et al. 2020). Concerningly, aspen in the Interior West is threatened by recent outbreaks of an invasive insect, oystershell scale (*Lepidosaphes ulmi* Linn.; hereafter OSS) (Crouch et al. 2021) (Fig. 1).

OSS is an armoured scale (Hemiptera, Diaspididae) that inserts its stylet through the bark of woody host plants to feed on the fluid of non-vascular cells (Griswold 1925; Beardsley and Gonzalez 1975). This feeding damages host cells, resulting in cell death and cracking of the host's bark under heavy infestations (Beardsley and Gonzalez 1975). Mortality of heavily infested branches, stems and stands may occur (Griswold 1925; Beardsley and Gonzalez 1975). OSS is polyphagous, with around 100 host genera globally (Miller and Davidson 2005b).



Figure 1. Photo of young aspen stand inside an exclosure (2 m tall fence built to exclude ungulates from browsing aspen) experiencing high levels of dieback and mortality from oystershell scale (OSS). Photos on the right show close-ups of OSS on aspen.

Twelve host genera (Acer, Alnus, Ceanothus, Cornus, Frangula, Fraxinus, Juglans, Lupinus, Populus, Ribes, Salix and Symphoricarpos) have been documented in the Interior West (Crouch et al. 2021). OSS is most common on woody, deciduous plants, especially those with relatively thin bark (Miller and Davidson 2005b). As an obligate parasite, OSS completes its entire life cycle on woody tissue of living hosts (Samarasinghe 1965; Crouch et al. 2024a). All OSS life stages are sessile, except for first instars (i.e. crawlers), which actively disperse along the host's stem or branches in search of a suitable feeding site (Miller and Davidson 2005b). Once a feeding site is found, OSS will remain there through adulthood (Griswold 1925). In sexually reproducing populations, males shed their tests and fly to mate with females; however, female-only populations occur and reproduce via parthenogenesis (Beardsley and Gonzalez 1975; Miller and Davidson 2005b). It is unclear which reproduction method occurs in the Interior West, although one observation suggests reproduction is predominately asexual (Cranshaw 2013). Questions also remain about OSS dispersal mechanisms and distances, but long-distance crawler dispersal is likely driven by human movement of infested plants, whereas shortand intermediate-distance dispersal are likely driven by wind, potentially with animal assistance (Griswold 1925; Beardsley and Gonzalez 1975; Magsig-Castillo et al. 2010). Crawlers do not actively move more than 1 m due to limited energy reserves and susceptibility to adverse climatic conditions (Beardsley and Gonzalez 1975; Magsig-Castillo et al. 2010).

Although the native range and introduction history of OSS are uncertain, the species was likely transported to North America by European settlers on infested plant material (Griswold 1925; Beardsley and Gonzalez 1975). OSS was first reported as a pest of apple trees (Malus spp.) in the 1700s and is now present throughout much of North America, especially in urban and ornamental settings (Griswold 1925; Miller and Davidson 2005b). Despite the species' polyphagous nature, its pervasiveness in North America and its long invasion history, OSS has rarely been a major pest in wildland forest settings (but see Sterrett [1915]; DeGroot [1967]; Houston [2001]). OSS was first reported on aspen in wildland forest settings in Arizona, USA by Fairweather (1992) and Zegler et al. (2012). In both cases, OSS abundance was low and impacts were minimal. However, in 2016, OSS was observed causing dieback and mortality of aspen in wildland forest settings (Grady 2017), and severe outbreaks have since been observed in both areas where Fairweather (1992) and Zegler et al. (2012) first observed OSS. Based on this invasion history, Crouch et al. (2021) suggested that OSS may be a sleeper species, which is defined as a non-native species that establishes successfully, but experiences slow population growth before suddenly awakening when conditions become favourable and experiencing rapid population growth (Groves 1999; Bradley et al. 2018; Frank and Just 2020). It is unclear what led to the awakening of OSS, but recent climatic changes, namely warmer and drier conditions (Williams et al. 2022), are a likely explanation (Crouch et al. 2021). A warming climate may have improved conditions for OSS by increasing the species' fitness and abundance and/ or by increasing susceptibility of aspen to infestation and mortality (Frank 2020; Crouch et al. 2021). OSS's role as a sleeper species and its potential interactions with climate change are concerning because continued climate warming may trigger OSS invasions in other areas of aspen's range (Crouch et al. 2021).

Critical to managing any invasive species is baseline information on its occurrence and impacts. The first peer-reviewed report of OSS outbreaks in aspen ecosystems indicated that OSS is already widespread in northern Arizona (Crouch et al. 2021). Crouch et al. (2021) also observed that OSS only occurred in the lower half of aspen's elevation range (< 2,533 m) and that OSS seemed to be particularly pervasive on and damaging to smaller recruiting stems (stems > 1.37 m tall and < 12.7 cm diameter at breast height [dbh; height = 1.37 m]). Specifically, mortality of recruiting stems was 5-10 times greater than that of overstorey trees in two sites with severe OSS infestations (Crouch et al. 2021). In a survey of aspen populations across Arizona, Crouch et al. (2024b) corroborated those early results by finding that there was significantly less OSS at higher elevations and that OSS was amongst the most important factors limiting aspen recruitment. Both studies also found that OSS was more common in fenced ungulate exclosures (2 m tall fences built to exclude ungulates from browsing on aspen) than outside of them. As recruitment is critical for self-replacement, these initial findings highlight the threat that OSS poses to aspen resilience and sustainability (DeRose and Long 2014; Crouch et al. 2023), warranting a comprehensive survey of OSS and its impacts on aspen in Arizona. We do not know which factors influence OSS's presence across the landscape and what specific impacts OSS has on aspen ecosystems. Obtaining this information is critical for informing management of OSS and mitigating damage caused by this high-impact invasive species. To fill these knowledge gaps, we sampled aspen populations across Arizona and addressed three questions: (1) What is the geographic extent of OSS in Arizona? (2) What impacts does OSS have on aspen? (3) Which biotic and abiotic factors influence the proportion of aspen stems infested by OSS?

Methods

Study area

Our study area encompassed aspen ecosystems across Arizona. Although OSS affects numerous hosts in Arizona (Crouch et al. 2021), our study focused on aspen because the first OSS outbreaks were observed in aspen ecosystems and because of aspen's importance as a foundation species. Aspen ecosystems in Arizona tend to be small (0.1-25 ha in size), especially compared to more northerly latitudes of aspen's range (Zegler et al. 2012). Aspen is limited to relatively high elevations (2,000-3,000 m), where lower temperatures and higher precipitation allow the drought-intolerant species to survive (Perala 1990; Rehfeldt et al. 2009). At lower elevations, small stands of aspen occur on north-facing slopes or in drainages with increased water availability, and as elevation increases, the aspen component tends to be more abundant and less aspect-limited (Rasmussen 1941; Zegler et al. 2012). In addition to occurring alone in small single-species stands, aspen commonly co-occurs with conifers, including ponderosa pine (Pinus ponderosa Lawson & C. Lawson var. scopulorum Engelm.) and Douglas-fir (Pseudotsuga menziesii [Mirb.] Franco var. glauca [Beissn.] Franco) at lower elevations, white pine (Pinus strobiformis Engelm. or Pinus flexilis James var. reflexa Engelm.) and white fir (Abies concolor [Gord. & Glend.] ex Hildebr.) at mid-elevations and corkbark fir (Abies lasiocarpa [Hook.] Nutt. var. arizonica [Merriam] Lemmon) and Engelmann spruce (Picea engelmannii Parry ex Engelm.) at the highest elevations.

Site selection

We sampled 220 aspen plots that represent the range of conditions under which aspen exists in Arizona (Fig. 2a). These plots were located across seven major areas: North Kaibab (n = 19), South Kaibab (n = 26), Flagstaff (n = 113), Mogollon Rim (n = 13), White Mountains (n = 25), Prescott (n = 17) and Coronado (n = 7) (Fig. 2a). All data were collected during the 2020, 2021 and 2022 growing seasons (June – October), when aspen trees had leaves.

To ensure that we obtained a representative sample of aspen sites and conditions, we stratified sites across four variables – elevation ($\leq 2,400$ m, > 2,400 m); aspect (north/east, south/west); ungulate management (none, fenced exclosure or jack-straw treatment [large piles of woody debris protecting aspen regeneration from ungulate browse]); and fire history (0–2 years post-fire, 2–20 years post-fire, > 20 years post-fire) – resulting in 24 strata. We first sought to obtain one plot for each stratum, which we accomplished for 21 of the 24 strata, before building out a sample that was proportional to how much aspen occurs in each stratum. We assessed aspen's actual occurrence in each stratum using an observed GIS layer of aspen's range on three national forest ranger districts surrounding Flagstaff (Flagstaff and Mogollon Rim Ranger Districts of the Coconino National Forest; Williams Ranger



Figure 2. Maps showing OSS presence and absence in **a** 220 study plots across seven major areas (in italics) where aspen occurs in Arizona, USA **b** study plots in the South Kaibab (left of green National Forest boundary line) and Flagstaff major areas (right of green line) and **c** study plots along the Mogollon Rim. These three areas are the only ones where OSS presence varied. OSS was present in all plots around Prescott and was absent in all plots in the North Kaibab, White Mountains and Coronado. Size of OSS presence circles is scaled, based on the proportion of aspen stems infested, with larger circles representing higher infestation rates.

District of the Kaibab National Forest) (DePinte 2018). Although this layer covers only three of the nine ranger districts we sampled, it is the most accurate estimation of where aspen occurs in Arizona because it is a fine-scale layer of aspen's recent presence, based on direct observations from an aircraft (DePinte 2018). We compared the proportion of aspen observed on the landscape, based on area from the GIS layer, to the proportion of aspen plots we sampled, based on the number of plots that fell into each stratum. We succeeded in obtaining a representative sample across elevation, aspect and fire history, with proportions of aspen observed in each stratum versus aspen sampled differing by less than 7% (Crouch et al. 2024b).

When possible, we prioritised re-measurement of existing aspen monitoring plots to reduce the number of redundant plots on the landscape and to facilitate research permission on national forest land. Specifically, we revisited plots previously established by the Coconino National Forest (n = 44), the Apache-Sitgreaves National Forest (n = 5), Zegler et al. (2012) on the Kaibab National Forest (n = 20) and Northern Arizona University's Ecological Restoration Institute on the San Francisco Peaks (n = 12). All four of these networks established plots using stratified or completely random sampling, ensuring the locations of these plots lacked bias. We established the remaining 139 plots by identifying aspen stands that filled target strata, standing on the edge of selected stands, laying out a linear transect longways through those stands and establishing plots every 30 m along the transects. The Coconino National Forest, Apache-Sitgreaves National Forest and Ecological Restoration Institute plots were also established along transects with plot spacings ranging from 100 m to 300 m. The Zegler et al. (2012) plots were established by randomly locating points within known aspen stands and sampling four plots in each cardinal direction 20 m from those points, resulting in plots that were spaced 28.3 m apart.

Field data collection

Each study plot consisted of two fixed-area, circular plots: an overstorey plot (8 m radius) and a nested regeneration plot (4 m radius) sharing the same plot centre (Zegler et al. 2012). We collected GPS coordinates at the centre of each study plot, recorded whether the plot fell in an area of ungulate management (i.e. fenced exclosure or jackstraw treatment) and noted whether there was evidence of recent conifer removal, as indicated by cut conifer stumps present in or directly adjacent to the plot. For a plot to be included in our study, it had to contain at least five live aspen stems between the 8 m overstorey and 4 m regeneration plots combined. In the 8 m overstorey plot, all trees with dbh > 12.7 cm were measured. In the 4 m regeneration plot, all trees > 0.02 cm in height and < 12.7 cm dbh were measured. In the regeneration plot, we classified stems into three size classes adapted from Zegler et al. (2012): short regeneration (< 1.37 m tall), tall regeneration (> 1.37 m tall and < 5.1 cm dbh) and saplings (5.1–12.7 cm dbh). We considered both tall regeneration and saplings to be recruiting stems (Crouch et al. 2024b). For all live aspen, we measured height, dbh (except for regeneration and recruits that were < 1 cm dbh), crown dieback ([percentage of dead branches above the bottom of the tree's live crown] 0%, 1-33%, 34-67%, > 67%), crown ratio (percentage of total tree height occupied by the live crown) and OSS presence and severity. We assessed OSS severity using the system devised by Crouch et al. (2021), which rates OSS severity on each tree from ground level to 6 m. Each tree's stem up to 6 m is divided equally into thirds, and severity is rated for each 2 m section (or shorter for trees

< 6 m tall) on both the north and south sides of the tree. Severity is rated 0-3: 0 = no OSS present, 1 = only a handful of OSS present (trace), 2 = OSS covers < 50% of section (light), 3 = OSS covers > 50% of section (severe). For every dead aspen and live tree species other than aspen, we recorded size class and dbh.

For all live aspen in our study plots, we documented the top three damaging agents present on each tree (Zegler et al. 2012). When more than three damaging agents were present, preference was given to agents with the greatest severity of impact (i.e. most likely to cause dieback and mortality) (Zegler et al. 2012). These damaging agents included insects, diseases, ungulate browse, other animal damage and abiotic damage. For insects and diseases, we grouped individual species into functional groups to facilitate analysis and because some biotic damage agents (e.g. defoliating insects) were impossible to identify based solely on damage signs and symptoms. These groups included sucking and gall-forming insects, bark beetles, wood-boring insects, defoliating insects, canker-causing diseases, foliar and shoot diseases and decay diseases (USDA Forest Service 2013; Steed and Burton 2015). The sucking and gall-forming insects group excluded OSS because we identified and measured OSS separately from other insects. We assessed certain cankers individually because of their potential to have outsized impacts on aspen tree health compared to less pathogenic diseases (Hinds 1985; Zegler et al. 2012; Crouch et al. 2023). The cankers we assessed individually were Cytospora canker (caused by Cytospora spp.), Hypoxylon canker (caused by Entoleuca mammatum [Wahlenb.] P. Karst), Ceratocystis canker (caused by Ceratocystis spp.) and sooty bark canker (caused by Encoelia pruinosa [Ellis & Everth.] Torkelson & Eckblad). We lumped all types of abiotic damage together, which included fire scarring of stems, foliar drought scorch and foliar chlorosis. We assessed animal damage to aspen stems, including browse, ungulate barking (i.e. elk chewing aspen bark) and other animal damage. We also indirectly quantified ungulate impacts by counting ungulate scat piles within the 8 m overstorey plot. We identified scat piles by species (i.e. elk [Cervus canadensis Erxleben], deer [Odocoileus hemionus Rafinesque or O. virginianus couesi Coues & Yarrow] or cattle [Bos taurus Linn.]) and treated piles from the same species as distinct when piles were clearly separated, contained more than three pellets and differed in colour or size (Bunnefeld et al. 2006; Rhodes and St. Clair 2018).

OSS extent

We assessed OSS presence and absence in each of the 220 study plots to determine the geographic extent of OSS in Arizona. We used descriptive statistics summarising the proportion of plots and trees infested by OSS to further explore geographic patterns of OSS invasions. We also assessed tree-level OSS severity across the four aspen size classes (i.e. short regeneration, tall regeneration, saplings and overstorey trees) to determine if there were differences in susceptibility to OSS. We analysed all data in R version 4.2.1 (R Core Team 2022), using the *dplyr* package (Wickham et al. 2022) for data manipulation and the ggplot2 package (Wickham 2016) for figure creation.

OSS impacts

We assessed OSS impacts on aspen at both the tree and stand levels. At the tree level, we built univariate regressions to quantify the influence of OSS presence and severity on aspen dieback and crown ratio, which are metrics that capture individual stem health (Schomaker et al. 2007). Tree-level OSS severity was calculated by taking the mean percentage of each category in the rating system (i.e. 1% for 1, 25% for 2 and 75% for 3) and calculating the mean for the six ratings recorded for each tree. We used the *nlme* package (Pinheiro et al. 2022) to fit four linear mixed-effects models with crown dieback and crown ratio as responses, OSS presence and severity as fixed effects and the hierarchical, nested structure of plots (i.e. plots [n = 220] within study sites [n = 87] within minor areas [n = 19] within major areas [n = 7]) as random effects. The random effects in these models and all others in our analysis were specified as random intercepts. Study site refers to a transect or group of plots that were spatially contiguous and occurred within the same strata, with distances between plots in the same site ranging from 30 m to 500 m. Minor area refers to a group of transects or plots in a larger, but still confined area (e.g. an individual mountain or fire footprint). To assess OSS impacts at the stand level, we built univariate regressions between aspen mortality and the proportion of aspen stems infested by OSS in each plot (hereafter referred to as OSS infestation rate). We used the *nlme* package (Pinheiro et al. 2022) to fit six individual linear mixed-effects models with the following response variables: dead aspen basal area, dead aspen density and density of dead aspen in each of the four size classes. We fitted OSS infestation rate as the fixed effect and the hierarchical, nested structure of plots (i.e. plots [n = 64], within study sites [n = 23], within minor areas [n = 9], within major areas [n = 4]) as a random effect. Sample sizes differ for these stand-level models compared to the tree-level models because we used only the 64 plots in which OSS occurred for these stand-level models.

Factors influencing OSS infestation rate

To assess drivers of OSS invasions in aspen ecosystems, we collected data representing an array of biotic (Table 1) and abiotic (Table 2) factors that may influence plot-level OSS infestation rate (i.e. the proportion of aspen stems infested by OSS). In total, we considered 99 variables across eight categories of potential influencing factors: stand structure, ungulates, other damaging agents, fire, management, site factors, soils and climate. We included as many potential influencing factors as possible because we had no prior data on which factors drive OSS invasions. Using tree diameter data, we calculated basal area of stems > 5.1 cm dbh for live aspen, all OSS host species and non-host species (Table 1). We calculated stem densities (trees ha⁻¹) for live aspen, all host species and non-host species across each of four stem size classes (i.e. short regeneration, tall regeneration, saplings and overstorey trees). Using the presence/absence data for all damaging agents on each live aspen stem, we calculated the proportion of stems affected by each agent in each plot (Table 1).

Using GPS coordinates collected at each plot's centre, we calculated elevation, aspect and slope using a 30 m² digital elevation model (Table 2). We transformed aspect into a continuous variable ranging from 0–2 with 0 representing southwest (225°) and 2 representing northeast (45°) (Beers et al. 1966). We also calculated heat load and potential annual direct radiation, two indices that assess site-level temperature based on slope, aspect and latitude (McCune and Keon 2002). We assessed fire occurrence in each plot for the past 20 years using wildland fire perimeters obtained from the USDA Forest Service Region 3 GIS database (https://www.fs.usda.gov/detail/r3/landmanagement/gis) and prescribed fire perimeters from national forest staff. We assessed fire severity using data obtained from the

Influencing factor	Mean	Std error	Range
Stand structure			
Aspen basal areaª	10.3	12.4	0–55.9
All hosts basal area	10.3	12.4	0–55.9
Non-host basal area	10.2	14.7	0-78.1
Aspen overstorey density ^b	172	254	0-1,194
Aspen sapling density	354	866	0–6,565
Aspen tall regeneration density	2,399	8,169	0-89,127
Aspen short regeneration density	8,694	17,345	0–136,873
All hosts overstorey density	172	254	0-1,194
All hosts sapling density	356	869	0-6,565
All hosts tall regeneration density	2,460	8,181	0-89,127
All hosts short regeneration density	8,745	17,332	0–136,873
Non-host overstorey density	115	168	0–945
Non-host sapling density	65	180	0-1,592
Non-host tall regeneration density	192	764	0–9,350
Non-host short regeneration density	1,394	3,992	0-33,224
Ungulates			
Browse ^c	0.30	0.31	0-1
Ungulate barking ^c	0.03	0.10	0-0.85
Total ungulate scat ^d	2.6	5.0	0–35
Elk (<i>Cervus canadensis</i>) scat	1.3	3.1	0–23
Deer (Odocoileus hemionus & O. virginianus couesi) scat	1.1	3.5	0–29
Cattle (Bos taurus) scat	0.3	1.7	0–20
Damaging agents ^c			
Sucking & gall-forming insects (excluding OSS)	0.09	0.14	0-0.80
Bark beetles	0.01	0.03	0-0.20
Wood-boring insects	0.22	0.21	0-0.83
Defoliating insects	0.60	0.27	0-1
Cytospora canker (caused by Cytospora spp.)	0.02	0.05	0-0.34
Hypoxylon canker (caused by Entoleuca mammatum)	0.002	0.011	0-0.10
Ceratocystis canker (caused by Ceratocystis spp.)	0.02	0.05	0-0.42
Sooty bark canker (caused by <i>Encoelia pruinosa</i>)	0.001	0.005	0-0.05
All cankers	0.33	0.26	0-1
Foliar & shoot diseases	0.19	0.24	0-0.94
Decay diseases	0.04	0.08	0-0.67
Other animal damage (excluding browse & barking)	0.01	0.02	0-0.15

Table 1. List of 33 biotic variables considered as potential influencing factors of plot-level oystershellscale (OSS) infestation rate. Plot-level (n = 220) mean, standard error and range are shown.

^a basal area = m^2 ha⁻¹

^b density = trees ha⁻¹

^c proportion of aspen stems affected by damaging agent

^d scat = pellet piles/plot

Monitoring Trends in Burn Severity programme (https://www.mtbs.gov/), which provides fire severity data at 30 m resolution. We created categorical variables to represent both fire occurrence and severity in addition to a binary variable for plots that burned twice in the past 20 years (Table 2). Finally, we used GPS coordinates and maps obtained from national forest staff to verify whether plots fell inside areas of ungulate management and conifer removal treatments, and we created binary variables for both ungulate management and conifer removal (Table 2).

Table 2. List of 66 abiotic variables considered as potential influencing factors of plot-level oystershell scale (OSS) infestation rate. Plot-level (n = 220) mean, standard error and range are shown for continuous variables, whereas percentage of plots in each category is shown for categorical variables.

Influencing factor	Mean	Std error	Range		
Damaging agents					
Abiotic damage ^a	0.01	0.05	0-0.61		
Fire		·			
Fire strata ^b		1 (14.1%), 2 (22.7%), 3 (63.2%)		
Fire severity ^c	1 (65.59	%), 2 (9.1%), 3	(11.4%), 4 (8.2%), 5 (5.9%)		
Burned twice ^d		0 (95.00	%), 1 (5.0%)		
Management					
Ungulate management ^e		0 (67.7%	%), 1 (32.3%)		
Conifer removal ^f		0 (87.7%	%), 1 (12.3%)		
Site factors					
Elevation (m above sea level)	2,543	237	1,976–3,038		
Aspect ^g	0.98	0.73	0–2		
Slope (°)	7.9	7.1	0.1–29.7		
Heat load (MJ/cm ² /yr)	0.98	0.07	0.71-1.08		
Radiation (MJ/cm ² /yr)	0.96	0.08	0.64–1.09		
Major area ^h	1 (3.6%), 2 (5	1.8%), 3 (6.4%)), 4 (9.1%), 5 (8.2%), 6 (11.8%), 7 1.4%)		
UTM easting	453804	77968	358542-674303		
UTM northing	3880092	89053	3589116-4052723		
Soils					
Soil order ⁱ	1 (2	2.7%), 2 (14.1%), 3 (13.2%), 4 (70.0%)		
Soil pH in H_2O (pH×10)	63.4	2.6	55.4–71.4		
Cation exchange capacity (CEC) (mmol(c)/kg at pH 7)	232.9	22.6	176.3–272.15		
Nitrogen (cg/kg)	110.0	22.5	80.0-188.3		
Soil organic carbon content (dg/kg)	135.2	22.8	93.8–193.9		
Bulk density (cg/cm ³)	147.5	5.8	130.1–157.8		
Sand content (g/kg)	321.7	85.6	187.5–592		
Clay content (g/kg)	269.1	51.2	129.7–397.7		
Volumetric fraction of coarse fragments (cm ³ /dm ³)	179.2	61.2	75.2–293.0		
Climate					
Degree-days below 0 °C	323.9	103.1	109.0–596.0		
Degree-days above 5 °C	1,883	377.7	1,215–2,819		
Degree-days below 18 °C	3,823	489.9	2,656–4,842		
Degree-days above 18 °C	137.1	94.4	24.5-389.0		
Degree-days above 10 $^{\circ}\mathrm{C}$ and below 40 $^{\circ}\mathrm{C}$	909.0	254.3	464.5-1,521.0		
Number of frost-free days	181.2	33.8	140.5–265.5		
Frost-free period	113.4	29.5	78.0–185.0		
Winter temperature (maximum) ^j	6.0	1.6	2.5-10.6		
Spring temperature (maximum)	13.8	1.4	10.7–17.8		
Summer temperature (maximum)	25.3	1.8	21.2–29.1		
Autumn temperature (maximum)	16.6	1.4	13.3–20.3		
Winter temperature (minimum)	-7.6	2.2	-10.82.2		
Spring temperature (minimum)	-1.2	2.0	-4.4–3.7		

Influencing factor	Mean	Std error	Range
Summer temperature (minimum)	9.1	2.1	6.5–14.0
Autumn temperature (minimum)	0.8	2.3	-1.7–6.2
Winter temperature (mean)	-0.8	1.8	-4.2-3.9
Spring temperature (mean)	6.3	1.6	3.2–10.4
Summer temperature (mean)	17.2	1.7	13.9–21.1
Autumn temperature (mean)	8.7	1.6	5.8-12.4
Precipitation as snow (annual) ^k	135.8	56.2	29.5-332.0
Winter precipitation ^k	211.3	75.0	83.5–516.5
Spring precipitation	148.8	34.8	66.5–240.0
Summer precipitation	147.8	69.9	64.5–292.5
Autumn precipitation	130.5	71.8	62.5-366.0
Winter relative humidity ¹	51.0	5.1	44.5–70.5
Spring relative humidity	51.7	3.8	47.0-66.0
Summer relative humidity	53.0	4.8	47.0-63.0
Autumn relative humidity	50.8	5.9	43.0-66.0
Winter Hargreaves reference evaporation ^k	30.0	39.6	0–125.5
Spring Hargreaves reference evaporation	272.1	25.0	192.5–323.0
Summer Hargreaves reference evaporation	463.1	31.2	396.5–535.0
Autumn Hargreaves reference evaporation	224.1	14.1	193.5–263.0
Winter climatic moisture deficit (CMD) ^k	6.2	8.2	0–26.0
Spring climatic moisture deficit (CMD)	163.6	25.0	117.0–216.0
Summer climatic moisture deficit (CMD)	325.6	76.3	177.0-461.5
Autumn climatic moisture deficit (CMD)	139.2	38.6	57.5–199.0
Winter climate moisture index (CMI) ^k	18.5	7.0	5.6-42.7
Spring climate moisture index (CMI)	-3.2	5.2	-14.7–9.6
Summer climate moisture index (CMI)	-29.9	10.3	-48.37.3
Autumn climate moisture index (CMI)	-11.0	8.0	-21.8-15.2
Annual dryness index ^m	0.07	0.02	0.04-0.12
Annual heat moisture index ⁿ	31.8	8.2	18.1–49.8
Summer heat moisture index ^o	118.8	52.1	45.1-242.4

^a proportion of aspen stems affected by damaging agent

^b categorical: 1 (0–2 yrs since fire), 2 (2–20 yrs since fire), 3 (> 20 yrs since fire)

^c categorical: 1 (unburned in past 20 yrs), 2 (unburned/low), 3 (low), 4 (moderate), 5 (high)

^d categorical: 0 (burned < 2 times in past 20 yrs), 1 (burned twice in past 20 yrs)

^c categorical: 0 (no ungulate management), 1 (exclosure or jackstraw)

^f categorical: 0 (no treatment), 1 (conifer removal)

 g 0–2 (0 = 225°, 1 = 135° or 315°, 2 = 45°)

^h categorical: 1 (Coronado), 2 (Flagstaff), 3 (Mogollon Rim), 4 (North Kaibab), 5 (Prescott), 6 (South Kaibab),

ⁱ categorical: 1 (Inceptisols), 2 (Mollisols – Borolls), 3 (Mollisols – Ustolls), 4 (Alfisols)

 j temperature = $^{\circ}C$

^k precipitation, evaporation, CMD, CMI = mm

¹ relative humidity = %

^m annual dryness index = annual degree days above 5°C ÷ annual precipitation

ⁿ annual heat moisture index = (annual temperature + 10) \div (annual precipitation \div 1,000)

° summer heat moisture index = warmest month temperature ÷ (summer precipitation ÷ 1,000)

Seasons for climate variables are winter (December – February), spring (March – May), summer (June – August), autumn (September – November).

^{7 (}White Mountains)

We obtained soils data from SoilGrids (https://www.isric.org/explore/soilgrids), which provides global soil mapping data at 250 m resolution (Poggio et al. 2021). We used 9 of 12 available soil metrics to capture variables that represent soil moisture (e.g. sand content and bulk density), fertility (e.g. cation exchange capacity, nitrogen and soil organic content), rooting environment (e.g. bulk density, clay content and coarse fragments) and chemical environment (e.g. soil pH) (Table 2). We aggregated mean values for each variable to a depth of 1 m because most lateral aspen roots occur within the first 1 m of the soil (Jones and DeByle 1985b). We obtained climate data from ClimateNA (https://climatena.ca/), which downscales PRISM data (Daly et al. 2008) at 800 m resolution (Wang et al. 2016). We expected climate to be an important driver of OSS infestation rate, so we included an array of climate variables that could potentially influence OSS and aspen. Specifically, we obtained variables representing annual and, when available, seasonal degree-days, temperature, precipitation, humidity, Hargreaves reference evaporation (hereafter evaporation) and drought for the two years preceding the date each plot was sampled (Table 2). We chose two years because repeated observations of OSS in the same field sites across multiple years indicated that most OSS we observed on trees had accumulated in the preceding two years (Crouch et al. 2024a). Although dead OSS are likely capable of lasting even longer on host trees, more recent climate data captures the most recent trends in OSS population growth.

We used random forests, structural equation modelling (SEM) and stand- and tree-level regressions to determine which biotic and abiotic factors drive OSS invasions. First, we used random forests to determine which of the 99 predictor variables had the strongest influence on plot-level OSS infestation rate. Random forests are a useful tool for assessing variable importance in regression and classification settings amongst an array of potential predictors (Breiman 2001). Specifically, we used the *VSURF* package (Genuer et al. 2015), which used 50 random forest runs, each of which was built using 2,000 trees, to rank predictor variable importance. *VSURF* is robust in noisy, high dimensional settings and in the presence of highly correlated predictors (Genuer et al. 2010). *VSURF* outputs a ranked list of variables, based on importance, which is calculated using out-of-bag mean square error for each tree. We used this ranked list of variables when building SEMs and assessing univariate relationships between influencing factors and OSS infestation rate. We also examined the top 10 climate variables in this list to search for climatic thresholds beyond which OSS does not occur in Arizona.

Once we obtained a list of variable importance from *VSURF*, we used SEM to assess how the most important predictors and their interactions affect OSS infestation rate. SEM is an insightful tool for ecological research because it allows the user to build models based on theoretical understanding of an ecological system, resulting in a network of causal, multivariate relationships with a complete accounting of direct and indirect relationships and the relative strengths of those relationships (Grace 2006; Lefcheck 2016). Our first step in building an SEM was to construct an *a priori* model, based on our theoretical understanding of how biotic and abiotic factors might influence OSS. This *a priori* model (Fig. 3) accounted for all 99 variables that potentially influence OSS infestation rate using the eight categories of influencing factors (i.e. stand structure, ungulates, other damaging agents, fire, management, site factors, soils and climate). We then built a "full" SEM, which included the highest ranked variable, based on random forests from each of the eight categories of influencing factors (Tables 1, 2). We used a combination of backward and



Figure 3. *A priori* structural equation model (SEM) illustrating hypothesised directional relationships amongst influencing factors and plot-level OSS infestation rate. Arrows indicate causal relationships, and colours correspond to each of the eight categories of influencing factors. See Tables 1, 2 for complete lists of measured variables included in each of these eight categories.

forward selection to optimise model fit (using AIC and Fisher's C statistic) and to maximise explanatory power (using \mathbb{R}^2 of the response variable). This optimisation process included removing variables with low significance in the model and adding in more than one variable per category (e.g. adding a second climate variable) when two variables from one category had high importance values, based on random forests. We also tested how swapping in one variable to replace another variable of the same category (i.e. replacing fire severity with fire strata) affected the model. To overcome issues with how SEM handles categorical predictors, we set categorical levels as numeric (i.e. 0 and 1 for binary variables or 0-k for ordinal variables with klevels) and modelled these variables numerically. We used the *piecewiseSEM* package to build SEMs because this package accommodates the use of mixed-effects models (Lefcheck 2016). For the individual regressions that underlie *piecewiseSEM*, we used the *lme4* package (Bates et al. 2015) to fit linear mixed-effects models with the hierarchical, nested structure of plots modelled as random effects.

We also fitted stand- and tree-level regressions to further assess how various factors influence OSS presence and severity. At the stand level, we took the top 25 factors influencing OSS infestation rate based on random forests and built univariate regressions to quantify relationship direction, strength and significance. We used the *nlme* package (Pinheiro et al. 2022) to fit linear mixed-effects models with plot-level OSS infestation rate as the response, the 25 individual influencing factors as fixed effects and the hierarchical, nested structure of plots as random effects. At the tree level, we built univariate regressions to determine the influence of aspen tree size on OSS presence and severity. We used the *nlme* package (Pinheiro et al. 2022) to fit eight linear mixed-effects models with OSS presence and severity as responses, with dbh, height, height-to-diameter ratio and size class as fixed effects and with the hierarchical, nested structure of plots as random effects. As size class is a categorical variable, we used the "anova" function in R (R Core Team 2022) to conduct one-way analysis of variance (ANOVA), allowing us to test for significant differences in OSS presence and severity amongst the four size classes. When ANOVA found a significant ($\alpha = 0.05$) difference between size classes, we used the *emmeans* (Lenth 2022), *multcomp* (Hothorn et al. 2008) and *multcompView* (Graves et al. 2019) packages to conduct post-hoc Tukey-adjusted pairwise comparisons and determine which size classes significantly differed. We used these same ANOVA procedures to compare height-to-diameter ratios inside versus outside areas of ungulate management to assess how these treatments influence aspen growth and, in turn, potentially OSS.

Results

Aspen condition

The mean total aspen basal area, including living and standing dead trees, in our 220 study plots was 14.6 m² ha⁻¹ (standard error [SE] = 1.0), of which dead trees made up 29.5% (Table 3). Mean total aspen density was 16,069 trees ha⁻¹ (SE = 1,304), 27.7% of which were dead. Looking at individual stem size classes, study plots contained an average of 239 overstorey aspen ha⁻¹ (SE = 20; 28.2% of which were dead), 519 saplings ha⁻¹ (SE = 79; 31.9% dead), 3,196 tall regeneration stems ha⁻¹ (SE = 626; 24.9% dead) and 12,115 short regeneration stems ha⁻¹ (SE = 1,587; 28.2% dead). Of the 9,965 live aspen stems we sampled, 34.2% of stems had no crown dieback, 44.5% of stems had 1–33% dieback, 13.9% of stems had 34–67% dieback and 7.5% of stems had 67–99% dieback (Table 3). Mean crown ratio of live aspen was 52.0% (SE = 0.5%).

Table 3. Summary data for aspen stand structure, crown condition and OSS presence. Means and standard errors of variables representing live and dead aspen basal area, live and dead aspen density in different stem size classes, live aspen crown ratio and dieback and OSS presence at the plot and tree levels. For categorical variables, percentage of plots or trees in each level are shown.

Variable	Mean	Std error			
Live aspen basal area (m ² ha ⁻¹)	10.3	0.8			
Dead aspen basal area (m ² ha ⁻¹)	4.3	0.4			
Total live aspen density (trees ha ⁻¹)	11,618.5	1,304.2			
Total dead aspen density (trees ha ⁻¹)	4,450.5	704.2			
Live aspen short regeneration density (trees ha ⁻¹)	8,693.8	1,169.4			
Dead aspen short regeneration density (trees ha ⁻¹)	3,420.9	674.6			
Live aspen tall regeneration density (trees ha-1)	2,399.1	550.8			
Dead aspen tall regeneration density (trees ha ⁻¹)	796.7	126.6			
Live aspen sapling density (trees ha ⁻¹)	353.6	58.4			
Dead aspen sapling density (trees ha ⁻¹)	165.5	42.6			
Live aspen overstorey density (trees ha-1)	172.0	17.1			
Dead aspen overstorey density (trees ha ⁻¹)	67.4	8.6			
Aspen crown ratio (%)	52.0	0.5			
Aspen crown dieback (categorical)					
0% dieback	34.2	0.5			
1–33% dieback	44.5	0.5			
34–67% dieback	13.9	0.3			
68–99% dieback	7.5	0.3			
Plot-level OSS presence (categorical)					
OSS absent	70.9	3.1			
OSS present	29.1	3.1			
Tree-level OSS presence (categorical)					
OSS absent	89.3	0.3			
OSS present	10.7	0.3			

OSS extent

OSS was present in 29% of study plots and occurred in four of seven major areas where aspen occurs in Arizona: South Kaibab, Flagstaff, Prescott and Mogollon Rim (Fig. 2a). OSS was not found in study plots we sampled in the North Kaibab, White Mountains or Coronado major areas. Prescott had the highest plot-level rate of infestation, with OSS present in all 17 plots. South Kaibab had 65.4% of plots infested (Fig. 2b), Mogollon Rim had 53.4% of plots infested (Fig. 2c) and Flagstaff had 20.4% of plots infested (Fig. 2b). Of the 9,965 live aspen stems we sampled, 10.7% were infested with OSS (Table 3). Tree-level rates of infestation across major areas were consistent with plot-level rates of infestation. When looking at all plots, not just those in which OSS occurred, Prescott had the highest proportion of trees infested (60.3%), followed by South Kaibab (20.8%), Mogollon Rim (16.2%) and Flagstaff (7.6%).

OSS infested aspen stems of all sizes, although there was a higher likelihood of infestation on trees taller than 1.37 m (i.e. tall regeneration stems and larger) (Fig. 4). Using the OSS severity rating, 6.9% of all live aspen stems we sampled had a mean rating of trace (only a handful of OSS present), 2.3% were light (OSS covers < 50% of measured tree surface) and 1.4% were severely infested (OSS covers > 50% of measured tree surface). In the 64 plots where OSS was present, mean severity ratings were 34.5% trace, 11.6% light and 7.2% severe. Patterns of OSS severity across stem size classes generally followed those of OSS presence, with more severe infestations occurring as rate of presence increased (Fig. 4).



Figure 4. OSS severity across four aspen stem size classes. Data shown were taken only from the 64 study plots in which OSS was observed and include only live trees. OSS severity was assessed using the rating system devised by Crouch et al. (2021), which rates OSS severity on each tree from ground level to 6 m. Each tree's stem up to 6 m is divided into thirds, and severity is rated for each 2 m section (or shorter for trees < 6 m tall) on both the north and south sides of the tree. The ratings are as follows: none (no OSS present), trace (only a handful of OSS present), light (OSS covers < 50% of section), severe (OSS covers > 50% of section).

OSS impacts

OSS presence at the tree level was significantly (p < 0.001) associated with reduced aspen crown ratio and increased crown dieback, based on univariate regression (Table 4). Trees infested with OSS were associated with a 9.8% reduction in crown ratio and a 0.5 unit increase in dieback compared to trees without OSS. Dieback was assessed on a categorical scale from 0 to 3 (0 = 0% dieback, 1 = 1-33% dieback, 2 = 34-67% dieback, 3 = 67-99% dieback), so a 0.5 unit increase on the categorical scale equates to a roughly a 16% increase in dieback. Tree-level OSS severity was also significantly (p < 0.001) associated with reduced crown ratio and increased dieback (Table 4). Tree-level OSS severity ranged from 0 to 0.75, with 0 indicating a tree without OSS and 0.75 indicating a tree with the highest possible severity rating (OSS covering > 50% of all six stem sections rated). Thus, an increase in OSS severity of 0.1 equates to a 10% increase in OSS infestation of the tree's stem up to 6 m in height. A 0.1 unit increase in OSS severity was associated with a 3.9% reduction in crown ratio and a 0.2 unit increase in dieback, which equates to roughly 6.6% dieback. Despite the high degree of significance for all four of these univariate relationships, OSS presence and severity explained a low proportion of the variance observed in aspen crown ratio and dieback ($R^2 \le 0.03$).

For the 64 study plots in which OSS was present, plot-level OSS infestation rate was significantly (p = 0.019) associated with increased dead aspen basal area (Table 5). An increase in OSS infestation rate of 1, which represents the difference between no stems infested by OSS and all stems infested by OSS, was associated with an increase in dead aspen basal area of 5.7 m² ha⁻¹. For context, mean basal area of living aspen in these 64 plots was $11.4 \text{ m}^2 \text{ ha}^{-1}$ (SE = 1.5) and in all 220 study plots was 10.3 m² ha⁻¹ (SE = 12.4). We also assessed the influence of OSS infestation rate on five measures of dead aspen density: total dead aspen and density of each of the four size classes. None of these univariate relationships was significant, although the tall regeneration (p = 0.054) and sapling (p = 0.061) models approached significance (Table 5). An increase in OSS infestation rate of 1 was associated with 1,654 more dead tall regeneration stems ha-1 and 532 more dead saplings ha-1. For context, there were 1,579 living tall regeneration stems ha^{-1} (SE = 167) and 867 living saplings ha⁻¹ (SE = 387) on average in the 64 plots where OSS occurred and 2,399 tall regeneration stems ha⁻¹ (SE = 8,169) and 354 living saplings ha⁻¹ (SE = 866) across all study plots. The models for total dead aspen density, density of dead short regeneration and density of dead overstorey trees were insignificant ($p \ge 0.350$).

Table 4. Univariate relationships between two measures of aspen stem health (crown ratio and dieback) and OSS presence and severity at the tree level.

Response	Predictor	Coefficient	Std error	<i>p</i> value	Marginal R ²
Aspen crown ratio (%)	OSS presence ^a	-9.83	1.25	< 0.001	0.012
Aspen crown ratio (%)	OSS severity (%)	-38.87	4.33	< 0.001	0.009
Aspen crown dieback ^b	OSS presence ^a	0.52	0.04	< 0.001	0.031
Aspen crown dieback ^b	OSS severity (%)	2.06	0.14	< 0.001	0.024

These relationships are based on linear mixed models. Marginal R^2 is based solely on the model's fixed effects, which were either OSS presence or severity. ^a 0–1 (0 = OSS absent, 1 = OSS present)

^b 0–3 (0 = 0% dieback, 1 = 1–33% dieback, 2 = 34–67% dieback, 3 = 68–99% dieback)

Table 5. Univariate relationships between six measures of dead aspen density and plot-level OSS infestation rate (i.e. proportion of stems infested by OSS). These models were fitted using data only from the 64 study plots in which OSS was present.

Response	Predictor	Coefficient	Std error	<i>p</i> value	Marginal R ²
Dead aspen basal areaª	OSS (%)	5.74	2.35	0.019	0.108
Total dead aspen ^b	OSS (%)	1,029.67	1,644.03	0.535	0.005
Dead aspen short regeneration ^b	OSS (%)	-646.59	961.49	0.505	0.005
Dead aspen tall regeneration ^b	OSS (%)	1,654.33	834.21	0.054	0.076
Dead aspen saplings ^b	OSS (%)	532.32	275.75	0.061	0.070
Dead overstorey aspen ^b	OSS (%)	33.15	35.04	0.350	0.015

These relationships are based on linear mixed models. Marginal R^2 is based solely on the model's fixed effect, which was plot-level OSS infestation rate.

^a m² ha⁻¹

^b trees ha

Factors influencing OSS infestation rate

We considered 99 potential factors influencing plot-level OSS infestation rate (i.e. proportion of aspen stems infested by OSS), and random forests indicated the five most important influences were autumn evaporation, elevation, degree-days between 10 °C and 40 °C, winter climate moisture index (CMI) and autumn precipitation (Table 6). Based on univariate relationships between the top 25 most important influencing factors and OSS infestation rate, the five strongest influencing factors were maximum winter temperature (R² = 0.43; *p* < 0.001), winter evaporation (R² = 0.41; *p* < 0.001), maximum spring temperature (R² = 0.30; *p* < 0.001), elevation (R² = 0.26; *p* < 0.001) and minimum spring temperature (R² = 0.21; *p* = 0.004).

The optimal SEM for plot-level OSS infestation rate (AIC = 861.6; Fisher's C = 1.018 with p = 0.907 [high p value indicates better fit]; response marginal R² = 0.53, conditional R² = 0.88 [marginal includes only fixed effects, conditional includes both fixed and random effects]) included seven influencing factors: autumn evaporation, winter CMI, maximum winter temperature, elevation, fire strata, live aspen sapling density and presence of ungulate management (Fig. 5). Based on this SEM, all influencing factors, except for winter CMI and elevation, had a significant (p < 0.05) direct effect on OSS infestation rate. Autumn evaporation had a negative direct effect (effect size = -0.33; p = 0.011) on OSS infestation rate, whereas maximum winter temperature (0.60; p = 0.011), fire strata (0.30; p < 0.001), live aspen sapling density (0.13; p = 0.001) and presence of ungulate management (0.26; p <0.001) had positive direct effects. Fire strata and ungulate management were categorical variables (Table 2), and SEM indicated that less recent fire and presence of ungulate management resulted in a greater OSS infestation rate. All three climate variables had significant ($p \le 0.003$) influences on fire strata, with recent fire being driven by less autumn precipitation, higher winter CMI and higher maximum temperatures in winter. Thus, more autumn evaporation indirectly led to more OSS, while higher winter CMI and maximum winter temperatures indirectly led to less OSS. More autumn evaporation and higher winter CMI also resulted in significantly ($p \le 0.003$) fewer aspen saplings, resulting in both climate variables having an additional negative indirect effect on OSS infestation rate. Finally, aspen sapling density was significantly (p < 0.001) lower at higher elevation, resulting in a negative indirect effect of elevation on OSS infestation rate.

Table 6. Relationships between OSS infestation rate and its most important influencing factors based on random forests. Top 25 most important variables influencing plot-level OSS infestation rate, based on 50 random forest runs, each of which was built using 2000 trees. Univariate relationships between influencing factors and OSS infestation rate were based on linear mixed models. See Tables 1, 2 for lists of all influencing factors considered.

	Random forests	Univariate regressions		
Rank	Influencing factor	Coefficient	Marginal R ²	<i>p</i> value
1	autumn evaporation	-0.003	0.014	0.195
2	elevation	< -0.001	0.263	< 0.001
3	degree-days 10-40 °C	< 0.001	0.197	0.003
4	winter CMI	0.005	0.015	0.302
5	autumn precipitation	0.002	0.168	0.002
6	winter evaporation	0.006	0.413	< 0.001
7	winter temp (max)	0.120	0.428	< 0.001
8	wood boring insects	0.121	0.007	0.034
9	winter precipitation	< -0.001	0.002	0.755
10	clay	< 0.001	0.003	0.638
11	degree-days < 0 °C	-0.001	0.190	0.003
12	snow	-0.002	0.146	0.001
13	spring temp (max)	0.105	0.301	< 0.001
14	spring CMD	0.004	0.109	0.002
15	spring evaporation	0.002	0.037	0.085
16	aspen saplings ha-1	< 0.001	0.018	0.001
17	host saplings ha ⁻¹	< 0.001	0.018	0.001
18	degree-days > 5 °C	< 0.001	0.198	0.003
19	spring temp (min)	0.071	0.210	0.004
20	host regeneration ha ⁻¹	< -0.001	< 0.001	0.455
21	UTM easting	< -0.001	0.088	0.170
22	other animal damage	0.070	< 0.001	0.891
23	summer temp (mean)	0.068	0.174	0.005
24	aspen regeneration ha-1	< -0.001	< 0.001	0.458
25	fire severity	-0.014	0.003	0.463

Marginal R² is based solely on the model's fixed effect, which was the influencing factor shown in each row.

To further assess the relationship between climate and OSS, we searched for thresholds using the top 10 climate variables that random forests indicated were the most important influences of plot-level OSS infestation rate. We identified clear elevational and climatic thresholds beyond which OSS does not occur in Arizona (Fig. 6). OSS was not observed in plots that exceeded 2,545 m in elevation, received greater than 152 mm of snow annually, experienced maximum winter temperatures below 5.25 °C, experienced maximum spring temperatures below 13.25 °C and had fewer than 825 degree-days between 10 °C and 40 °C (calculated as accumulated temperature difference from the degree-day threshold, rather than a true accumulation of degree-days [Wang et al. 2016]). In addition, OSS was not observed in plots with less than 218 mm of autumn evaporation, except for one plot which had 202.5 mm of evaporation and a single aspen stem infested with OSS.

We also assessed univariate relationships between tree-level OSS presence and severity and four measures of aspen stem size: size class, height, dbh and



Figure 5. Optimal SEM for OSS infestation rate that minimised AIC and maximised response R^2 . Significant (p < 0.05) path coefficients are shown in bold, and their corresponding paths are depicted as solid lines. In contrast, insignificant coefficients are not in bold, and their corresponding paths are shown as dashed lines. Path thickness indicates strength of its coefficient, with wider paths indicating stronger relationships.

height-to-diameter ratio. One-way ANOVA indicated that there were significant differences (p < 0.001) in OSS presence and severity amongst the four stem size classes (Table 7). OSS presence was significantly greater on overstorey trees and saplings compared to tall and short regeneration, and OSS presence was significantly greater on tall regeneration than short regeneration. Saplings and tall regeneration had significantly greater OSS severity than overstorey trees and short regeneration, while overstorey trees had significantly greater OSS severity than short regeneration. OSS presence and severity significantly (p < 0.001) increased with increasing height and decreasing dbh (Table 7). In addition, OSS presence and severity significantly ($p \le 0.042$) increased with increasing aspen height-to-diameter ratio, also known as slenderness, and one-way ANOVA indicated that height-to-diameter ratios were significantly (p < 0.001) greater inside areas of ungulate management than outside these areas. Similar to the tree-level crown ratio and dieback results, aspen stem size explained a low proportion of the variance observed in OSS presence and severity (marginal $\mathbb{R}^2 \le 0.02$) (Table 7).

Discussion

OSS extent

OSS is widely distributed throughout aspen ecosystems in central Arizona (Fig. 2a), confirming the initial report of OSS outbreaks in the region (Crouch et al. 2021). This study added plots in three areas not surveyed by Crouch et al. (2021) – North Kaibab, White Mountains and Coronado – none of which had OSS.



Figure 6. Elevation and climate thresholds beyond which OSS does not occur in aspen ecosystems in Arizona. Relationships between plot-level OSS infestation rate and **a** elevation **b** snowfall **c** autumn evaporation **d** degree-days above 10 °C and below 40 °C **e** maximum winter temperature and **f** maximum spring temperature. Red lines indicate thresholds above or below which OSS does not occur in aspen ecosystems in Arizona.

Response	Predictor	Coefficient	Std error	<i>p</i> value	Marginal R ²
OSS presence ^a	size class SR	-12.246 c	1.936	< 0.001	0.014
	TR	1.752 b	0.189	< 0.001	-
	S	2.905 a	0.279	< 0.001	-
	0	2.904 a	0.280	< 0.001	
OSS severity (%)	size class SR	0.010 c	0.008	0.239	0.021
	TR	0.027 a	0.002	< 0.001	
	S	0.035 a	0.004	< 0.001	-
	0	0.011 b	0.003	< 0.001	-
OSS presence ^a	height (m)	0.006	0.001	< 0.001	0.004
OSS severity (%)	height (m)	0.001	< 0.001	< 0.001	0.002
OSS presence ^a	dbh (cm)	-0.003	0.001	< 0.001	0.005
OSS severity (%)	dbh (cm)	-0.001	< 0.001	< 0.001	0.012
OSS presence ^a	height:diameter (m)	0.458	0.166	0.006	0.001
OSS severity (%)	height:diameter (m)	0.114	0.056	0.042	0.001

Table 7. Univariate relationships between tree-level OSS presence and severity and four measures ofaspen stem size (size class, height, dbh and height-to-diameter ratio).

These relationships are based on linear mixed models. Aspen size class abbreviations: SR (short regeneration, < 1.37 m tall), TR (tall regeneration, > 1.37 m tall and < 5.1 cm dbh), S (saplings, 5.1–12.7 cm dbh), O (overstorey trees, > 12.7 cm dbh). Different letters after coefficients indicate significant differences amongst size classes, based on post-hoc Tukey-adjusted pairwise comparisons. Marginal R^2 is based solely on the model's fixed effects, which was aspen size class, height, dbh or height-to-diameter ratio.

^a 0–1 (0 = OSS absent, 1 = OSS present)

We also added plots in the South Kaibab, Flagstaff, Prescott and Mogollon Rim major areas, identifying more sites where OSS occurs in these areas than were initially reported (Grady 2017; Crouch et al. 2021). Aspen ecosystems we sampled in the North Kaibab and White Mountains may be free of OSS because wetter, colder climates in those areas (Suppl. material 1) may be unsuitable for OSS, as we discuss later. All plots we sampled in both these areas were above 2,545 m in elevation, which was the threshold above which we did not observe OSS anywhere in Arizona (Fig. 6a). Alternatively, these areas may have evaded OSS invasions for a different, unknown reason. For example, OSS spread at the landscape scale is likely facilitated by its ability to infest a wide array of hosts in addition to aspen (Miller and Davidson 2005b; Crouch et al. 2021). However, our ability to determine how other host species facilitate OSS invasions of aspen ecosystems is limited due to the lack of fine-scale mapping of those species' ranges, particularly understorey hosts, such as Ceanothus spp. Unlike the North Kaibab and White Mountains, aspen ecosystems on the Coronado tend to occur on relatively warmer, drier sites (Suppl. material 1), but OSS may have been absent in our Coronado study plots because they were all located in areas burned the previous year by the 2020 Bighorn Fire. Importantly, our sampling was not exhaustive and was restricted to aspen ecosystems, so OSS may occur in the North Kaibab, White Mountains and Coronado. Although some of our study plots contained non-aspen tree species that are OSS hosts (Table 1), few of these were infested, and our monitoring did not account for presence of understorey hosts. More extensive monitoring in the areas where we did not find OSS, including on hosts other than aspen, is warranted.

Prescott had the highest rates of OSS infestation of the seven major areas we studied, with 100% of plots (n = 17) and 60.3% of live aspen in the region being

infested. This is concerning because Prescott also had the highest levels of sustainable aspen recruitment, defined as the number of recruits needed for successful self-replacement of the existing overstorey (Crouch et al. 2024b), so OSS outbreaks in this area might eventually counteract successful recruitment. One reason why there is so much aspen recruitment in Prescott is because elk populations, which significantly inhibit aspen recruitment (Beschta and Ripple 2010; Fairweather et al. 2014; Crouch et al. 2023, 2024b), do not occur in the area. Therefore, ungulate exclosures, which also do not exist in the area, do not facilitate OSS invasions of aspen ecosystems in Prescott as they seem to do in other areas of Arizona. Instead, Prescott may have more OSS than other areas because it has been present in the area for longer. The earliest report of OSS occurring on wildland aspen in Arizona was in Prescott approximately 30 years before our study occurred (Fairweather 1992). Another possible explanation for why OSS is so abundant in Prescott is because aspen sites in this area tend to experience warmer temperatures than aspen in other parts of Arizona (Suppl. material 1). As we discuss below, warmer temperatures are a significant driver favouring OSS.

OSS impacts

OSS negatively affected aspen health at both the tree and stand levels. Aspen trees infested with OSS had significantly lower crown ratios and higher dieback, indicating reduced stem health. As OSS infestations became more severe, crown ratio significantly decreased, and dieback significantly increased (Table 4). Despite the significance of these observed effects, OSS explained a low proportion of variance in aspen crown ratio and dieback ($R^2 \le 0.03$), indicating that OSS is one of many factors influencing aspen health. Crown damage was likely caused by the feeding behaviour of OSS, which extracts fluid from the host plant's non-vascular cells (Griswold 1925; Beardsley and Gonzalez 1975). Although crown dieback is a common symptom associated with feeding of armoured scales on the bark of woody hosts, the exact mechanism that causes crown damage is not well understood (Miller and Davidson 2005a). One exception is San Jose scale (Diaspidiotus perniciosus Comstock), for which feeding on woody tissue has been shown to impair growth of affected cells and alter the number, size and arrangement of xylem and phloem cells (Beardsley and Gonzalez 1975). If similar cell damage is caused by OSS feeding, then crown damage may be the result of impaired water transport and increased vulnerability to embolism (Hillabrand et al. 2019). OSS might also damage aspen by reducing photosynthetic capacity because aspen bark is photosynthetic (Jones and DeByle 1985b). Alternatively, OSS feeding may compromise stem health by causing trees to allocate resources from growth to defence (Cope et al. 2021). At the stand level, plots with more stems infested by OSS had significantly greater dead aspen basal area, with a 10% increase in OSS infestation rate equating to an increase of 0.57 m² ha⁻¹ in dead aspen basal area (Table 5). Although density of total dead aspen stems and density of dead stems in each of the four size classes had insignificant relationships with OSS infestation rate, the models for dead tall regeneration and saplings approached significance, suggesting that these intermediate-sized, recruiting stems may be more susceptible to OSS than regeneration and overstorey trees (Crouch et al. 2024b).

Density of aspen saplings and saplings of all host species were significantly associated with increased plot-level OSS infestation rate (Table 6), providing additional evidence that intermediate-sized, recruiting stems seem to be most susceptible to OSS. At the tree level, OSS infestations were more severe on tall regeneration and saplings and were associated with taller and thinner stems (Table 7). We hypothesise that short regeneration had such low infestation rates because these stems remain shorter than 1.37 m for only a few years (Jones and Schier 1985), which reduces the likelihood of OSS finding them before they grow into taller size classes. Short regeneration may also be more likely to evade OSS because they are smaller targets. We hypothesise that overstorey trees had lower OSS severity than tall regeneration and saplings because overstorey trees tend to have thicker bark, which may inhibit OSS's ability to feed on larger aspen stems. For example, Just et al. (2020) hypothesised that thick bark of older red maples (Acer rubrum L.) may inhibit feeding of gloomy scale (Melanaspis tenebricosa Comstock). On the other hand, overstorey trees may have had lower OSS severity than intermediate-sized stems because the OSS severity rating system does not assess OSS above 6 m in height. The mechanisms underlying OSS's outsized impacts on intermediate-sized, recruiting stems merit further research, but the potential impacts of this finding are clear. Recruiting stems are critical indicators of aspen community resilience (Rogers and Mittanck 2014; Rogers 2017), and increased mortality of these stems from OSS poses a major threat to sustainability of aspen ecosystems (Crouch et al. 2023, 2024b).

Factors influencing OSS infestation rate

Climate was the most important factor driving OSS invasions of aspen ecosystems in Arizona. According to random forests, seven of the top 10 and 15 of the top 25 factors influencing OSS infestation rate were climate variables. Moreover, SEM indicated that climate variables, namely autumn evaporation and maximum winter temperature, had the strongest direct effect on infestation rate. Generally, warmer and drier conditions were associated with increased OSS. For example, greater OSS infestation rate was associated with fewer degree-days below 0 °C and more degree-days between 10 °C and 40 °C, with warmer temperatures in winter, spring and summer, with less winter precipitation and annual snowfall and with greater spring climate moisture deficit (CMD) and winter evaporation (Table 6). The strong relationship between climate and OSS infestation rate is not surprising because of the well-established influence of climate, particularly temperature, humidity and precipitation, on development of armoured scale insects (Beardsley and Gonzalez 1975). For example, degree-day models are commonly used to predict armoured scale development (Miller and Davidson 2005a), and warmer temperatures tend to increase development rate, survival and fecundity of scale insects (Frank 2020 and references therein).

Alternatively, the relationship between climate and OSS might be mediated through host stress (Crouch et al. 2021), as indicated by the water and drought stress variables we found to be important, such as precipitation, evaporation, CMI and CMD. The plant stress hypothesis would suggest that drought not only weakens aspen defences but also increases nutritional quality, leading to increased OSS fitness and abundance (White 1984; Dale and Frank 2017; Frank 2020). In support of this hypothesis, the optimal host for sap-feeding herbivores is one that has experienced long-term, intermediate drought stress punctuated by temporary releases from that stress (Kolb et al. 2016). Our findings seem to align with this hypothesis because, although arid conditions in general were associated with more OSS, we

found that wetter conditions in autumn (e.g. reduced evaporation and increased precipitation) were associated with an increased OSS infestation rate. Therefore, we hypothesise that OSS thrives when conditions are consistently arid with temporary releases from drought in autumn. Further research is needed to assess this hypothesis and elucidate the mechanisms underlying climate's influence on OSS.

Although previous research has indicated that elevation is an important limiting factor for OSS (Crouch et al. 2021, 2024b), our study revealed that climate drives this relationship. Based on SEM, elevation did not have a significant direct effect on plot-level OSS infestation rate when climate was accounted for in the model. Instead, climate variables had significant direct effects on OSS infestation rate and were significantly correlated with elevation. A clear elevation threshold was observed in our study, in which no OSS was found above 2,545 m (Fig. 6a). However, thresholds were also observed for climate variables (Fig. 6b–f), indicating that there are cooler, wetter climatic conditions which are currently unsuitable for OSS and these conditions are closely correlated with elevation. We suspect that these thresholds, particularly the elevation threshold, will change as the climate continues to warm (Seager et al. 2007); however, repeated measurements of study plots are required to confirm this hypothesis.

Given OSS's hypothesised role as a sleeper species and the strong influence of climate on OSS infestation rate, our study suggests that climate change caused OSS population sizes to rapidly increase and to transition from an innocuous pest to a high-impact invasive species. We have shown that OSS is associated with more arid conditions. Therefore, we hypothesise that prolonged, record drought and warmer temperatures over the past 10-20 years (Williams et al. 2022) caused OSS populations in Arizona to awaken. Climate is generally considered the most common cause of sleeper species awakenings (Bradley et al. 2018; Frank and Just 2020), although other possible explanations exist, such a new mutualism, evolution, loss of a predator or parasite or introduction of a new genetic strain (Lockwood et al. 2005; Borden and Flory 2021; Spear et al. 2021). A new mutualism is unlikely because there are no documented mutualists associated with OSS (Griswold 1925; Miller and Davidson 2005b), whereas the other explanations are conceivable and represent fruitful areas for future research. For example, armoured scale populations are susceptible to suppression by natural enemies (Edmunds 1973; Raupp et al. 2010; Frank 2020), so release from one or more natural enemies could lead to OSS population growth. Interestingly, climate change can cause such a release by creating asynchrony between phenology of scale insects and their natural enemies (Frank 2020). Research on OSS genetics is needed to determine whether evolution or introduction of a new genetic strain of OSS may have influenced awakening of OSS populations. Due to the strong relationship between climate and OSS, we have serious concerns that OSS populations in other areas will continue awakening with continued climate warming (Seager et al. 2007), as has recently been observed in Utah, Nevada and Idaho (Williams 2021; Grady et al. 2022).

In addition to climate, fire had a strong influence on OSS. Fire strata was the third most important direct influence on OSS infestation rate based on SEM. Less recent fire resulted in significantly more OSS, suggesting that fire can be an important strategy for managing OSS. Of the 31 study plots that experienced fire in the two years prior to sampling, only two plots were infested with OSS, and the infestation rate in these two plots was low, with only 1.7% and 7.3% of aspen stems infested. In contrast, 40 of the 139 plots that had not experienced fire in the preceding 20 years were infested with OSS. Fire may be an important limiting

factor for OSS because it kills OSS both directly and indirectly, by killing hosts upon which OSS is dependent (Crouch et al. 2021). Aspen has thin bark, making it highly susceptible to fire mortality, so even low severity fire can kill overstorey aspen (Jones and DeByle 1985a; Stoddard et al. 2018) and, in turn, the OSS feeding on aspen. Alternatively, lack of OSS in areas of recent fire might simply be due to timing. It might take two or more years after a fire for stands of recruiting stems, which we know are susceptible to OSS infestation, to develop.

We also found that ungulate management strategies, which primarily consisted of fenced exclosures, resulted in significantly more OSS (Fig. 5). Although exclosures are highly effective at promoting aspen recruitment (Crouch et al. 2024b), this study supports previous evidence that OSS is more prevalent in areas of ungulate management (Crouch et al. 2021, 2024b). We hypothesise that ungulate exclosures promote OSS by directly increasing aspen density and, potentially, by indirectly reducing host vigour. Dense aspen stands provide more available host material and feeding sites for OSS and might facilitate OSS spread. High densities might also reduce host vigour through increased inter-tree competition (Ashton and Kelty 2018), thereby making individual aspen stems more susceptible to OSS. In support of this hypothesis, we found that aspen inside areas of ungulate management had greater height-to-diameter ratios compared to aspen outside these areas. Dense stands produce stems with greater height-to-diameter ratios (i.e. increased slenderness) (Wang et al. 1998; Frey et al. 2004), likely because trees prioritise height growth over diameter growth to compete with their neighbours for sunlight. We expect that this is especially true for shade-intolerant species such as aspen (Perala 1990). Frey et al. (2004) hypothesised that slender aspen in dense stands are more susceptible to stressors because they have more difficulty with hydraulic conductivity and may be more vulnerable to water stress and reduced photosynthesis as the stand naturally self-thins. In our study, aspen with greater height-to-diameter ratios (i.e. taller, thinner stems) were significantly associated with increased OSS presence and severity (Table 7), indicating that slender stems with reduced vigour may be more susceptible to OSS. Research is needed to determine which of these mechanisms explains why more OSS is found inside ungulate exclosures. For example, Lindroth et al. (2023) found reduced levels of phenolic glycosides, which is aspen's primary chemical defence against ungulate and insect herbivory, in unbrowsed aspen found inside exclosures, so perhaps exclosures alter aspen growth-defence trade-offs in a way that increases susceptibility to OSS.

Management implications

OSS is already widespread across several States in the Interior West, including Arizona, so management tactics intended to eradicate this pest are unlikely to succeed. Eradication is further complicated by OSS's ability to infest an array of different host species and by the fact that small populations are exceedingly difficult to detect due to OSS's small size and cryptic colouring (Crouch et al. 2021). Instead of eradication, management resources may be better spent suppressing OSS population sizes, mitigating damage to native ecosystems through integrated pest management and developing slow-the-spread tactics (Sharov et al. 2002). Robust monitoring to assess an invasive species' extent and impacts is a critical first step for successful management. Continued monitoring in areas of Arizona where we did not find OSS (e.g. southern Arizona, North Kaibab and White Mountains) is necessary, as is continued monitoring of areas where OSS already occurs to document potential changes to the species' range (i.e. will OSS migrate to higher elevations in the future?). We also recommend monitoring of OSS in nursery stock of all host species, in urban areas that contain aspen, in riparian areas that connect urban and wildland aspen populations and in wildland aspen ecosystems, all of which may harbour sleeper populations of OSS (Frank and Just 2020; Crouch et al. 2021). Our findings can help managers identify stands at risk and prioritise which aspen ecosystems to monitor. For example, OSS is most likely to occur on warmer, drier sites, in areas that have not recently experienced fire and in stands that have dense recruitment, such as those inside fenced ungulate exclosures. Specifically, the climatic and elevation thresholds we identified (Fig. 6) can guide monitoring efforts. Although elevation is the easiest metric for managers to consider when searching for OSS, the 2,545 m threshold is liable to change across aspen's expansive range. Instead, the climate thresholds are likely to be more consistent outside our study area, though we anticipate such climate-based thresholds will shift as warming continues. These climate data can be easily obtained via ClimateNA (https://climatena.ca/).

Our findings can also be used to guide management that seeks to suppress OSS populations and mitigate damage to aspen ecosystems. Our study indicates that three strategies might help to suppress OSS populations: (1) increasing application of fire at the landscape scale, (2) reducing reliance on ungulate exclosures and (3) decreasing aspen stand density. Fire has a negative influence on OSS, and although frequency and size of wildfires will likely continue to increase as climate warming continues (Seager et al. 2007; Singleton et al. 2019), managers may consider implementing prescribed fire to suppress OSS infestations in individual aspen stands. Frequent, low-severity fire may be detrimental to aspen stands, especially in the presence of chronic ungulate browse (Crouch et al. 2023), so fire should be implemented with caution and infrequency when managing for OSS. Advantages of fire as an OSS management strategy include low cost of implementation, the ability to kill OSS on multiple host species including understorey plants and the fact that fire is a natural component of aspen ecosystems (Crouch et al. 2021). Prescribed fire may also be an effective strategy for slowing the spread of OSS from newly-discovered invasion sites.

Another strategy that managers may consider is reducing use of fenced ungulate exclosures. Reducing reliance on exclosures should help reduce OSS population sizes, although this will require finding other ways to overcome chronic ungulate browse that threatens aspen ecosystem resilience, adaptive capacity and sustainability (Rogers 2017; Crouch et al. 2023, 2024b). Dense stands with abundant recruiting stems are the structure that seems most favourable for OSS population growth. However, it remains unclear whether the relationship between aspen stand density and OSS is due to increased host availability, facilitated spread amongst densely growing stems and/or decreased host vigour in more slender stems. Further research is needed to determine how stand density and growth versus defence trade-offs influence aspen susceptibility to OSS at different spatial scales.

A third strategy managers may consider for suppressing OSS populations is reducing aspen stand densities via thinning. Thinning might also promote aspen resistance to drought, as reduced growth rates which occur in dense stands are associated with increased mortality during drought (Kane and Kolb 2014; Ireland et al. 2014, 2020; Crouch et al. 2023). However, thinning must be implemented with care in aspen stands because aspen is sensitive to mechanical damage from logging equipment and susceptible to subsequent infection by canker-causing fungi and decay diseases (Walters et al. 1982; Jones and Shepperd 1985). Another potential drawback to thinning is that sudden, direct exposure of aspen stems to sunlight may cause sunscald (Krasnow et al. 2012), creating potential infection courts for pathogens. Before we can wholeheartedly recommend use of prescribed fire or silvicultural strategies like thinning to manage OSS, long-term experimental research is needed to assess the efficacy of these strategies for suppressing OSS populations and mitigating damage to aspen ecosystems. Research is also needed to assess the efficacy of other forms of management, such as biological control and application of systemic insecticides, that have no direct negative effect on aspen and will be critical components of an integrated pest management programme for OSS. Our future work seeks to address these needs by assessing OSS and aspen responses to various suppression strategies.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: RWH, KMW, CDC, AMG, MMM, NPW. Data curation: CDC, KMW. Formal analysis: CDC. Funding acquisition: MMM, CDC, AMG, KMW, NPW. Investigation: CDC, AMG, KMW, NPW. Methodology: MMM, CDC, AMG, NPW, RWH, KMW. Project administration: AMG, CDC, NPW, KMW. Resources: NPW, AMG, KMW. Supervision: KMW. Validation: CDC. Visualization: CDC, KMW. Writing – original draft: CDC. Writing – review and editing: CDC, MMM, KMW, NPW, RWH, AMG.

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Data availability

All of the data that support the findings of this study, including R code, are available via the Environmental Data Initiative (https://doi.org/10.6073/pasta/bd7be772e435ed0ba5585aae5a96f3e7).

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Supplementary material 1

Means and standard errors for the 92 continuous variables considered as potential influencing factors of plot-level OSS abundance, summarized across each of the seven major areas in our study

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Research Article

Low impact of *Carpobrotus edulis* on soil microbiome after manual removal from a climate change field experiment

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Abstract

Synergic effects between climate change and invasive species may alter soil microbial diversity and functioning, as well as cause major shifts in physicochemical properties. Moreover, some of these ecological impacts may manifest even after the removal of the invasive species. We have conducted a field experiment to assess such effects on soil microbial communities (fungi and bacteria) and physicochemical properties seven months after the removal of Carpobrotus edulis (L.), an invasive plant of coastal dune ecosystems. C. edulis grew on the experimental plots for 14 months under current ("invasive species treatment") and increased warming and drought conditions ("combined treatment"). Then, all plant parts (above and belowground biomass) were removed with a non-aggressive eradication method and soil samples were collected seven months later in the experimental and control plots (no invasive species and current climatic conditions). We predicted a general compositional shift in microbial communities in response to the presence of the invasive species. Moreover, given that water is the most limiting factor in this type of ecosystem, we also predicted a more pronounced compositional shift in the treatment combining invader presence and climate change. While species richness was similar amongst treatments, we observed some taxonomic and functional variation in soil microbial communities. Notably, fungal and bacterial communities exhibited contrasting responses. The species composition of bacteria differed significantly between the "invasive species" and "control" treatments, while, in the case of fungi, the most substantial difference occurred between the "invasive species" treatment and the combined treatment of "invasive species and climate change". Some chemical properties, such as carbon and nitrogen content or pH, strongly differed amongst treatments, with the "invasive species" showing a different response compared to the other two treatments. Overall, our study suggests smaller short-term effects on the microbial community compared to soil chemical properties. Furthermore and contrary to our initial expectations, the potential impact on the soil microbiome seemed to be weaker in the face of rising temperatures and drought conditions predicted by climate change. This outcome highlights the remarkable complexity of the impact of invasive species and climate change on belowground microbial communities.

Key words: Climate warming, coastal ecosystems, drought, global change, invasive species, soil microbial community



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Introduction

Synergic interactions between climate change and invasive species are considered a major driver of biodiversity loss (Walther et al. 2009), with subsequent potential impact on ecosystem functioning (Pyšek et al. 2020; Isbell et al. 2023). Invasive plant species are detrimental to biodiversity because they reduce native plant diversity (McGeoch et al. 2010; Pyšek et al. 2012; Castro-Díez et al. 2016) and alter soil properties and biota (van der Putten et al. 2007; Vilà et al. 2011; Zhang et al. 2019) through aboveground-belowground feedback loops (Reinhart and Callaway 2006; van der Putten et al. 2013). Plant-soil feedbacks are critical elements of ecosystems, with plants shaping soil physicochemistry, as well as the composition and functioning of microbial communities (Wardle et al. 2004; Ehrenfeld et al. 2005). In turn, soil microbiota can affect plant growth directly through negative (i.e. pathogens and root herbivores) and positive (mutualists) interactions (van der Putten et al. 2007; Ehrenfeld 2010). Hence, disturbance-induced shifts in soil communities, such as those driven by plant invasions, may lead to critical changes in soil structure and functioning despite the seemingly high functional redundancy of soil biodiversity (Custer and van Diepen 2020; Torres et al. 2021). Some of these ecological impacts, mostly those associated with indirect effects on soil and plant communities and/or soil properties, may even persist after the removal of the invasive species (i.e. residual or legacy effects, Corbin and D'Antonio (2012)), as reported by Parsons et al. (2020). Moreover, an additional layer of complexity is introduced by climate change, which will likely further disrupt plant-soil interactions (van der Putten et al. 2016; Pugnaire et al. 2019; Trivedi et al. 2022) and may also affect the magnitude and duration of invasion legacies (Albertson et al. 2024). Therefore, synergic effects between climate change and invasive species may extend beyond promoting their establishment in new ranges (Walther et al. 2009) to influencing ecosystem responses and resilience. In a global change context, understanding invasive species effects on important ecosystem components, such as the soil microbiome, even after their eradication, is critical to forecast their shortand medium-term impacts under current and future climate conditions.

Plant invasions alter soil structure, chemistry and biota by introducing shifts in litter quality, root growth and root exudates (Liao et al. 2008; Custer and van Diepen 2020; Torres et al. 2021). These changes in microbial communities and soil properties are considered important mechanisms that facilitate the establishment and expansion of invasive species in their new ranges (Reinhart and Callaway 2006; Rodríguez-Echeverría 2010; Lorenzo and Rodríguez-Echeverría 2012; Dawson and Schrama 2016). Moreover, climate change also exerts a direct impact on soil microbiomes (Jansson and Hofmockel 2020). Raising temperatures may stimulate metabolic rates, promoting growth and diversity of soil communities, while limited water availability may have the opposite effect, reducing microbial biomass and biodiversity (van der Putten et al. 2016; Pugnaire et al. 2019; Jansson and Hofmockel 2020). Hence, an increase in aridity due to climate change is expected to reduce the diversity and abundance of soil bacteria and fungi (Maestre et al. 2015). However, our understanding on how plant-soil feedbacks may disrupt ecosystem functioning under future environmental conditions is still very limited (Classen et al. 2015; van der Putten et al. 2016; Anthony et al. 2020) and even more in the case of interactions with invasive plant species. Regarding the sole impact of invasive species on soil microbial communities, previous meta-analyses have reported both highly heterogeneous, but
limited impacts on soil microbiomes (Custer and van Diepen 2020), an increase in microbial activity (Xu et al. 2022), as well as a tendency of bacterial diversity to increase with the presence of herbaceous invasive species (Torres et al. 2021). To move towards an integrative assessment of the impact of different global change drivers on the soil microbiome, field experiments simulating species invasion under increased warming and drought conditions offer great opportunities to assess the synergic impact of invasive species and climate change on soil microbial communities.

Coastal dune ecosystems are fragile and vulnerable to plant invasions (Castillo and Moreno-Casasola 1996; Muñoz-Vallés and Cambrollé 2013). One of the most aggressive invaders of these habitats worldwide is Carpobrotus edulis (L.) N.E. Br., a mat-forming species that inhibits germination and reduces light, space and nutrients for native plants (Campoy et al. 2018). While the impacts of C. edulis are locality-specific (Novoa et al. 2014; Rodríguez-Caballero et al. 2020), the invasion often results in an increase in organic matter, salinity, humidity and available phosphorus (P) and nitrogen (N) (Santoro et al. 2011; Novoa et al. 2013; Vieites-Blanco and González-Prieto 2018a; Rodríguez-Caballero et al. 2020). Concurrently, soil pH and the availability of other macronutrients, such as calcium (Ca), sodium (Na) or magnesium (Mg), typically decrease in invaded areas, although increases or no significant changes on these soil characteristics have also been reported (Vilà et al. 2006; Conser and Connor 2009; Novoa et al. 2013; Novoa et al. 2014; Vieites-Blanco and González-Prieto 2018a; Caravaca et al. 2022). These changes in soil properties and nutrient cycling have been linked to alterations in the structure and function of microbial biota (Badalamenti et al. 2016; Novoa et al. 2020; Rodríguez-Caballero et al. 2020; Caravaca et al. 2022) and, in fact, plant-soil feedbacks have been identified as an important mechanism promoting C. edulis invasion (de la Peña et al. 2010; Vieites-Blanco and González-Prieto 2018b). Furthermore, the recovery of native vegetation is hampered by legacy effects on soil abiotic properties, such as the presence of allelopathic substances in *C. edulis* litter (Conser and Connor 2009; Novoa et al. 2012) or the accumulation of carbon and nitrogen compounds, which may favour the establishment of ruderal species (Novoa et al. 2013). However, soil properties might recover to pre-invasion conditions (Novoa et al. 2013) and, given the strong link between soil properties and soil microbiota, it could be hypothesised that this recovery would be also mirrored by microbial communities in the soil.

In this study, we assess the effects on soil abiotic and biotic components seven months after C. edulis removal from a field experiment in which it had grown under current and future climate conditions for 14 months (see Suppl. material 1: appendix 1 for details and photographs of this experiment). A previous study showed that C. edulis growth and physiological performance increased under warming and drought, suggesting that invasion would be promoted under climate change (Campoy et al. 2021). Here, we focus on the effects on soil microbiome and physicochemistry observed several months after C. edulis specimens were removed from experimental plots. We predict a general compositional shift in microbial communities in response to the presence of the invasive species and, in particular, that this shift would be observable after the removal of the invasive species. Moreover, given that water is the most limiting factor due to reduced soil water-holding capacity in this type of ecosystem, we anticipate a more pronounced compositional shift in the treatment combining invader presence and climate change. Thus, we expect the combined effects of the invasive species and climate change would cause a stronger impact on soil microbial communities.

Materials and methods

Experimental design

The study was conducted on an experimental field plot located over a secondary dune in the island of Sálvora, within the National Park of the Atlantic Islands (northwest of the Iberian Peninsula). From September 2015 to November 2016 (14 months), a full factorial experiment was performed in forty subplots $(1.55 \text{ m} \times 1.75 \text{ m})$ to study the responses of the invasive *C. edulis* to climate change (Campoy et al. 2021). The duration of the experiment is four times longer than the one considered in a previous greenhouse experiment, which reported effects of C. edulis on the microbiome after three months (de la Peña et al. 2010). In detail, C. edulis was transplanted on to the experimental subplots in September 2015. At the end of this experiment (November 2016), all plant parts (above and belowground biomass) were carefully harvested (i.e. hand-pulling) and no further intervention was carried out until soil sample collection (June 2017). At the time of harvest, C. edulis was the dominant species, in terms of dry mass contribution, in the plant community, both in the invaded subplots [Inv], where it accounted for 28% of the community and in the climate change subplots [Inv_ClimCh]), where it accounted for 47% of the community. For the current study, 24 subplots were considered with the following treatments: eight control subplots (uninvaded), where spontaneous, natural vegetation had grown under current climatic conditions; eight subplots where C. edulis had grown under current climatic conditions (i.e. invasive species treatment [Inv]) and eight subplots where C. edulis had grown under modified temperature and rainfall conditions (i.e. combined treatment: invasive species + climate change [Inv_ClimCh]), according to the predictions for the study area (EEA 2017), specifically 2 °C temperature increase and 33% rainfall decrease. For detailed information of the experimental set-up, see Campoy et al. (2021) and the additional description provided in Suppl. material 1: appendix 1).

To assess differences in soil microbiome amongst treatments, we took five samples from the top 2–10 cm of soil in each subplot with a soil sampler that was disinfected with 10% diluted bleach. The top 2-cm layer was discarded in order to remove recently accumulated organic material not integrated into the soil structure. To avoid cross-contamination, the first sample from each subplot was discarded to ensure that no traces of disinfectant or soil from the previous subplot remained in the soil sampler. The other four samples were mixed to make a composite sample with which we filled two 2 ml sterilised cryogenic vials. The vials were immersed in a container with liquid nitrogen until their arrival at the laboratory, where they were stored at -80 °C until sample processing for genomic analyses.

Analysis of soil properties

In each of the 24 subplots, we also took one soil sample from the top 2–10 cm, as described in the previous section, for soil physico-chemical analyses. Samples were sieved (< 2 mm) and divided into fresh subsamples, which were kept at 4 °C for inorganic N measurements, and air-dried subsamples, which were finely ground (< 100 μ m) in a planetary ball mill (Retsch PM100, Germany, with cups and balls of zirconium oxide) for the rest of the analyses.

Soil pH was measured in a 1:2.5 soil:solution ratio, both in water and 0.1 M potassium chloride (KCl), with a pH-meter (Metröhm, Switzerland). Electrical con-

ductivity (EC) was determined in soil extracts (1:5 soil:water ratio) with an EC meter (Metröhm, Switzerland). Total soil C and N and the molar ${}^{15}N/{}^{14}N$ ($\delta^{15}N$) and $^{13}C/^{12}C$ ($\delta^{13}C$) ratios were determined in an elemental analyser (Carlo Erba, Milano, Italy) coupled to an isotope ratio mass spectrometer (Finnigan Mat, delta C, Bremen, Germany). Nitrogen isotope ratios were analysed because they can provide valuable tracers for biogeochemical cycles susceptible to be affected by invasion at local scales (Liao et al. 2008). To this end, $\delta^{15}N$ has been proposed as an integrator of N cycling and used to show the magnitude of the effect of plant invasion in soil N dynamics and soil microbial communities (Sperry et al. 2006; Hellmann et al. 2016). Organic C and δ^{13} C were also measured after calcium carbonate (CaCO₂) removal with the 'capsule method', using 20% hydrochloric acid (HCl) (Brodie et al. 2011). Soil 3 (Eurovector, Milano, Italy), an elemental reference material and the isotope standards IAEA-CH-6 and IAEA-CH-7, for δ ^{13}C and IAEA-N1 and IAEA-N2, for δ ^{15}N (from the International Atomic Energy Agency, Vienna, Austria) were included in each set of 10 samples to check the accuracy of the results. Nutrients available in the soil and trace elements (Al, Ca, Cu, Fe, K, Mg, Mn, Na, P and Zn) were extracted with a mixture of 1 M NH₄Ac (ammonium acetate) and 0.005 M DTPA (soil:solution ratio of 1:5). The mixture stirred for 2 h, was filtered through cellulose paper (Filter- Laboratory 1242, Ø 90 mm) and analysed with a simultaneous ICP-OES (Varian Vista Pro, Mulgrave, Australia) (García-Marco et al. 2020). Inorganic N species (i.e. NH_{4}^{+} and NO_{3}^{-}) were extracted with 2 M KCl (1:5 soil:solution ratio) and measured by microdiffusion, as described by Vieites-Blanco and González-Prieto (2018a).

DNA isolation, amplification and sequencing

DNA was isolated using the DNeasy PowerSoil Pro DNA isolation kit (Qiagen), strictly following the manufacturer's instructions and including an extraction blank to check for cross-contamination. For fungi, a fragment of the ITS1 genomic region (of around 360 bp) was amplified using the primers ITS1F (Gardes and Bruns 1993) and ITS2 (White et al. 1990). For bacteria, a fragment of the 16S rRNA genomic region (of around 300 bp) was amplified using the primers 515F (Parada et al. 2016) and 806R (Apprill et al. 2015). Illumina sequencing primers were attached to these primers at their 5' ends. Supreme NZYTaq 2x Green Master Mix (NZYTech) was used for DNA amplification. The oligonucleotide indices required for multiplexing different libraries in the same sequencing pool were attached in a second limited-cycle PCR. A negative control that contained no DNA (BPCR) was included in every PCR round to check for contamination during library preparation. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Then, libraries were pooled in equimolar amounts and sequenced in a fraction of a NovaSeq PE250 lane (Illumina). DNA quantification was done with Qubit (ThermoFisher). A detailed description of the DNA extraction, amplification and sequencing is provided in the Suppl. material 1: appendix 2. DNA metabarcoding analyses were carried out by AllGenetics & Biology SL (www.allgenetics.eu).

Processing of sequencing data

The obtained amplicon reads were processed using QIIME 2 (release 2021.2) (Bolyen et al. 2019). DADA2 (Callahan et al. 2016), implemented in QIIME 2, was used to remove the PCR primers, filter the reads according to their quality,

denoise, merge the reads, remove the chimeric reads and cluster the resulting sequences into amplicon sequence variants (ASVs).

Non-biological DNA (primers, indices and sequencing adapters) at the reads ends was trimmed with cutadapt (Martin 2011). To remove low-quality data, fungi reads were further truncated at position 191 (forward) and at position 216 (reverse) after checking the read quality profiles. In the case of bacteria, reads were truncated at position 202 (forward) and 187 (reverse). Error rates were learned from the dataset to denoise, using the parametric error model implemented in DADA2. To reduce computation time, reads were dereplicated and the R1 and R2 reads were merged with DADA2 by overlapping a minimum region of 12 identical base pairs. As a final step, chimeras were removed by the DADA2 pipeline. The number of sequences which passed the different DADA2 processing steps are shown in Suppl. material 1: table S1.

Taxonomy was assigned to ASVs, based on the UNITE reference database (Abarenkov et al. 2020) (updated in May 2021) for fungi and the SILVA reference database (Quast et al. 2013) (updated in August 2020) for bacteria using the pretrained classifier of each respective database and the feature-classifier classify-sklearn approach (Bokulich et al. 2018) implemented in QIIME 2. To avoid misrepresented ASVs and mistagging, singletons (i.e. ASVs containing only one-member sequence in the whole dataset) occurring at a frequency below 0.01% in each sample were removed. Sequences assigned only at the kingdom level ("Fungi" or "Bacteria" and "Archaea", respectively) and the unidentified sequences were removed. ASVs present in the negative controls that were assigned to bacteria/fungal taxa, were also removed. Additionally, in the case of bacteria, non-bacterial ASVs were removed.

All metabarcoding bioinformatic analyses were carried out by AllGenetics & Biology SL (www.allgenetics.eu).

Statistical analyses

The final filtered ASV table was converted into a Biological Observation Matrix file (.biom) and directly imported into R 4.1.2 (R-Core-Team 2021) using the package phyloseq (McMurdie and Holmes 2013). Sample 34 was removed from further analysis given that preliminary inspection of the data showed its anomalous behaviour in terms of soil properties and community composition.

To test for differences in soil properties amongst experimental treatments, we computed a multivariate analysis of variance (MANOVA) using Pillai's Trace as test statistic with the manova() function. Given the large number of soil variables, we summarised soil variables into Principal Components using function principal() with "varimax" rotation from the psych package (Revelle 2023) prior to MANO-VA analyses. When needed, we transformed variables to approach normality and only PCA components accounting for at least 70% of cumulative variance were retained. Function powerTransform() in car package (Fox and Weisberg 2019) was used to guide boxcox transformations.

To test for differences in the number of ASVs (observed richness) and, independently, in read depth amongst treatments, we fitted Negative Binomial Generalised Linear Models (NB GLM) with function glm.nb() in MASS package (Venables and Ripley 2002) for fungi data and, independently, for bacteria data. We also assessed whether richness relationships may be affected by an additive or multiplicative effect of read depth by sequentially comparing these GLM models with function anova() and a χ^2 test. Additionally, we checked that estimates of rarefied richness to the smallest read depth provided highly similar values to observed richness (Suppl. material 1: fig. S1) and analogous results in the NB GLM models. We computed rarefied richness with function rarefy_even_depth() in phyloseq package and averaged it across 100 resamples.

To test for differences in community composition amongst treatments, we computed a Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) with function adonis2() in the vegan package (Oksanen et al. 2023). To quantify differences in community composition, we first centre-log ratio- (CLR) transformed the data using function transform() in microbiome package (Lahti and Shetty 2012-2019). With CLR-transformed data, we used a Euclidean distance in the adonis2() function, which is equivalent to Aitchison distance, the metric recommended for this type of data (Gloor et al. 2017). We used function gg_ordiplot() in ggordiplots package to represent differences in community composition amongst treatments and function betadisper() in vegan to assess the homogeneity of dispersions (variances) amongst treatments. To assess which treatments were significantly different, we also conducted pairwise PERMANOVAs, considering only two treatment categories at a time. We complemented the analysis of community composition with a Differential Abundance Analysis (DAA) at the genus level, in order to assess whether some particular taxa differed in their relative abundance amongst treatments. We used function trans_diff() in microeco library (Liu et al. 2020) with the ANCOMBC (Lin and Peddada 2020) and ALDEx2 (Fernandes et al. 2014) methods. Differences in ALDEx2 were assessed with the Kruskal-Wallace and the glm tests. Both ANCOMBC and ALDEx2 take into account the compositional nature of the data and provide consistent results across studies (Nearing et al. 2022). Moreover, ALDEx2 uses the CLR- transformation, as in the PERMANOVA analyses.

To assess whether soil properties would explain differences in diversity (richness and community composition) once the "Treatment effect" is accounted for, we followed a manual stepwise selection procedure on the richness models and, independently, on the community composition models. For richness models, we considered treatment as a random effect and fitted a Generalised Linear Mixed-Effects model (GLMM) for the negative binomial family with function glmer.nb() in lme4 package (Bates et al. 2015). In each step, we assessed which variable had the largest additional significant contribution to the reduced model by using function anova(). For community composition models, we used the argument "by = margin" implemented in adonis2() to independently assess the marginal effects of each term to the reduced model.

To assess whether microbial functional profiles differed amongst treatments, we assigned functional traits to the obtained ASVs using FUNGuild (Nguyen et al. 2016) in the case of fungi and FAPROTAX (Louca et al. 2016) in the case of bacteria with function trans_func() in microeco package. FUNguild ranks the results within a confidence ranking as "highly probable", "probable" and "possible". Only those results marked as highly probable or probable were used, as recommended by Nguyen et al. (2016). Fungi were ascribed to one of the three main trophic groups (symbiotroph, saprotroph and pathotroph), as well as minor categories (e.g. dung saprotrophs) and bacteria were classified according to 45 functions related to energy source, N-cycling or C-cycling. Functional groups with an average relative abundance above 1% were tested for differences amongst treatments using MANOVA.

All analyses were performed in R (R Core Team 2021). The R code is provided as supporting information (Suppl. material 2).

Results

Composition of microbial communities

We delimited a total of 1295 ASVs of fungi and 5793 ASVs of bacteria (Table 1). The mean richness per sample was 256 for fungi and 1150 for bacteria. Rarefaction curve plots suggest that samples were complete (Suppl. material 1: fig. S2), despite read depth being significantly lower in the control treatment than in the global change treatments in the case of bacteria (negative-binomial likelihood ratio stat (NB LR stat) = 7.77, p = 0.020; Fig. 1). Marginally significant differences in read depth amongst treatments were also observed for fungi data (NB LR stat = 5.90, p = 0.052).

Fungal diversity was represented by 11 phyla, with Ascomycota being the dominant phylum with over ca. 90% of reads, followed by Basidiomycota and Mortierellomycota (Fig. 2, Suppl. material 1: fig. S3). The remaining phyla had lower representation (< 1%). In the case of bacteria, 34 different phyla were detected (Fig. 2), with 12 phyla with relative abundance above > 1%. Actinobacteria, Proteobacteria and Acidobacteria were the dominant bacteria phyla accounting for 70–75% of the total reads. A large number of classes (Fig. 2, Suppl. material 1: fig. S3) and orders (Suppl. material 1: fig. S4) were represented in the data (Suppl. material 1: fig. S4). Notably, the proportion of ASVs that were found in all treatments was relatively low (fungi = 28.9%; bacteria = 28.3%), but corresponded to a large proportion of reads (fungi = 92.9%; bacteria = 87.6%), thus evidencing that the dominant ASVs were found in all treatments (see Suppl. material 1: fig. S5 for details).

Differences in soil properties

Variability in soil properties was summarised into six principal components, accounting for 75.5% of variance (Suppl. material 1: table S2). Variable transformations and loadings can be found in the Suppl. material 1: table S2. A MANOVA analysis showed significant differences amongst treatments (Pillai–Bartlett statistic = 1.208; $F_{12,32} = 4.06$, p < 0.001) in PCA components related to carbon and nitrogen compounds, pH, EC and available Ca and Mn (Table 2). In general terms, the invasive species treatment was the one showing a distinct type of behaviour, while the control and the combined treatments (Inv_ClimCh) had similar soil properties (Fig. 3). Differences in raw variables of soil properties are shown in Suppl. material 1: fig. S6.

Table 1. Summary statistics of the number of reads (read depth) and ASVs.

	Fungi	Bacteria			
Reads					
Mean (\pm SD) per library	71544.8 ± 14898.0	76349.7 ± 14304.1			
Range per library	44717; 106444	48801; 104709			
Mean per ASV	279.7 ± 749.0	66.4 ± 94.0			
ASVs					
Total	1295	5793			
Mean per library	255.8 ± 23.2	1150.0 ± 139.1			
Range per library	220; 318	893; 1439			



Figure 1. Differences in read depth (**a**, **c**) and observed ASVs richness (**b**, **d**) of fungi and bacteria amongst treatments. Boxplot represents the median value and the interquartile range (IQR). Whiskers extend to \pm 1.5 * IQR. Outliers (i.e. data beyond the end of the whiskers) are plotted individually. Significance letters are based on negative binomial GLM contrasts (p < 0.05). Note that the ASV richness analysis for bacteria was only marginally significant (p = 0.056) and significant differences amongst treatments disappeared when sequencing depth is accounted for. Treatment: Control; Invasive species (Inv); Invasive species and climate change scenario (Inv_ClimCh).



Figure 2. Differences in taxonomic composition (relative abundance of ASVs corresponding to different phyla [**a**, **b**] or classes [**c**, **d**]) amongst treatments. Differences in taxonomic composition amongst samples are shown in the Suppl. material 1: fig. S3). Taxonomic abundance was computed with function trans_abund() in microeco package. Treatment: Control; Invasive species (Inv); Invasive species and climate change scenario (Inv_ClimCh).



Figure 3. Boxplot of the differences in soil properties amongst treatments. Soil properties have been summarised into Principal Components after varimax rotation (Rotated Component, RC). Only components contributing to ≥ 0.7 cumulative variance after varimax rotation are retained for further analyses. Main variable loadings for each component can be found in Table 2. Boxplot represents the median value and the interquartile range (IQR). Whiskers extend to $\pm 1.5 *$ IQR. Outliers (i.e. data beyond the end of the whiskers) are plotted individually. Significance letters are based on TukeyHSD tests (p < 0.05). Treatment: Control; Invasive species (Inv); Invasive species and climate change scenario (Inv_ClimCh).

Table 2. ANOVA tests after multivariate analysis of variance (MANOVA, Pillai–Bartlett statistic = 1.208; $F_{12,32} = 4.06$, p < 0.001) of differences in soil properties amongst treatments. Soil properties have been summarised into Principal Components after varimax rotation (Rotated Component, RC). Soil variables with large loading (≥ 0.70) are shown for each component. A full table of PCA variable loadings is provided in the Suppl. material 1: table S2.

Rotated component	F-value	р	Loadings (≥ 0.70)
RC1	4.3	0.028	$\rm NH_4^{\ *}, \rm NO_3^{\ -},$ Total N, Total C, Inorganic C, Organic C*, EC*
RC9	7.1	0.004	Ca, Mn
RC2	5.0	0.017	pH KCl, pH H ₂ O
RC3	0.03	0.970	K, Na*
RC7	12.1	< 0.001	δ ¹³ C _{total} * (-)
RC5	0.8	0.453	C/N* (-)

*Variable transformed prior to PCA analysis, see Suppl. material 1: table S2.

Differences in species richness and community composition

Species richness was not significantly different amongst treatments in fungi (NB LR stat = 3.48, p = 0.176) and only marginally significant in bacteria (NB LR stat = 5.75, p = 0.056) (Fig. 1). In fact, the treatment effect turned to be not significant when an additive model with sequencing depth was considered in bacteria, as

the number of reads was the only significant predictor in such model (Z = 16.39, p < 0.001, see Suppl. material 1: table S3 for details). Analogous models for averaged rarefied richness provide identical results and are shown in Suppl. material 1: table S3.

We also built a Generalised Linear Mixed-Effects model (GLMM) with treatment as a random effect in order to assess if soil PCA components had a significant contribution when included into this minimal model. In the case of fungi, only RC9 (with Ca and Mn as main variables loadings) had a significant contribution, with an increase in explained variance of 3.2% (Suppl. material 1: table S4). Soil PCA components did not significantly improve model fitting once treatment was included as a random effect in a GLMM of bacteria richness (Suppl. material 1: table S4).

PERMANOVA analyses evidenced differences in community composition amongst treatments both for fungi ($F_{2,20} = 1.37$, p = 0.001) and bacteria $(F_{2,20} = 1.16, p = 0.036)$, although the proportion of explained variance was low in both cases (fungi = 12.0%; bacteria = 10.4%, see ordination in Fig. 4). Pairwise PERMANOVA evidenced that, in the case of fungi, the combined treatment (Inv_ClimCh) was significantly different from both the invasive ($F_{1,14} = 1.60$, p = 0.002) and control treatments ($F_{1,13} = 1.35$, p = 0.008), but the latter two did not significant differ ($F_{1,13} = 1.16$, p = 0.175). Regarding bacteria, only the invasive and control treatments were significantly different ($F_{1,13} = 1.24$, p = 0.037), as no significant differences were observed amongst the rest (Control vs. Inv_ClimCh: $F_{1,13} = 1.18$, p = 0.073; Inv vs. Inv_ClimCh: $F_{1,14} = 1.07$, p = 0.216). The data did not show heterogeneity in dispersion amongst treatments (betadisper: fungi: $F_{2,20} = 1.99$, p = 0.163; bacteria: $F_{2,20} = 0.349$, p = 0.710). Despite the observed differences in community composition, differential abundance analyses (DAA) evidenced few cases of significant differences at the genus level. In fact, ALDEx2 did not detect any significant difference, while ANCOMBC showed significant differences in eight fungal genera and three bacteria genera, all of them with low relative abundances (Suppl. material 1: table S5, figs S7, S8).



Figure 4. Ordination plot representing differences in community composition amongst treatments. Differences in community composition are computed based on Aitchison distance after Centre-Log Ratio (CLR) transformation of the data. Principal components are extracted with function rda() in library vegan and represented with function gg_ordiplot() in library ggordiplots. Treatment: Control; Invasive species (Inv); Invasive species and climate change scenario (Inv_ClimCh).

We also conducted a manual stepwise procedure to assess if soil PCA components significantly contributed to explaining differences in community composition once the treatment factor was accounted for. The final model included the RC1 (mainly N and C variables) and RC2 (pH) components in the case of fungi and the RC2 (pH) and RC3 (K, Na) components in the case of bacteria (Suppl. material 1: table S4). These components notably increased the explained variance to 24.5% in fungi and 23.9% in bacteria.

In the analysis of fungal functional diversity, 55.1% (n = 714) of ASVs were assigned to a trophic group with "Highly Probable" or "Probable" confidence. Saprotrophs were the most abundant group in all treatments, followed by pathotrophs and symbiotrophs (Suppl. material 1: table S6). Wood and soil saprotrophs were the most represented guilds within saprotrophs, while plant pathogens and animal pathogens were the most abundant within pathotrophs. We observed significant differences in the proportion of reads assigned to each trophic group in each treatment (Pillai–Bartlett statistic = 1.62; $F_{26,18} = 2.96$, p = 0.010). The relative abundance of saprotrophs was higher in the invasive and combined treatments than in the control (ANOVA $F_{2,20} = 6.96$, p = 0.005, Fig. 5). There was also higher relative abundance of dung saprotrophs in the combined treatment than in the invasive one (ANOVA $F_{2,20} = 3.9$, p = 0.038, Fig. 5). No significant differences were observed in the other categories



Figure 5. Differences in relative abundance of trophic groups amongst treatments. Only main trophic groups and those where significant differences were observed are shown. Boxplot represents the median value and the interquartile range (IQR). Whiskers extend to $\pm 1.5 *$ IQR. Outliers (i.e. data beyond the end of the whiskers) are plotted individually. Significance letters are based on TukeyHSD tests (p < 0.05). Functional groups were assigned with FUNGuild (Nguyen et al. 2016) and only assignments with "Highly Probable" or "Probable" confidence were considered. The variable "Dung saprotroph" was log-transformed for MANOVA analysis. Treatment: Control; Invasive species (Inv); Invasive species and climate change scenario (Inv_ClimCh).

(Fig. 5, Suppl. material 1: fig. S9). Moreover, two genera identified as having lower differential abundance (ANCOMBC test) in the combined treatment (Inv_ClimCh), *Boeremia* and *Coniella*, were classified as plant pathogens (Suppl. material 1: fig. S7).

In the case of bacteria, 48 functional traits were assigned to a total of 1111 ASVs (19.3% of total ASVs), although most functions had a low representation with average relative abundance < 1%, except in the case of chemoheterotrophy (mostly aerobic chemoheterotrophy), nitrate reduction and dark hydrogen oxidation, see Suppl. material 1: table S7). All bacterial functions related to N-cycling were represented albeit in low percentages. Amongst these, bacteria with nitrate reduction ability was the group with highest relative abundance, followed by nitrogen fixation. Fermentation was the most represented function within the C-cycle. No significant differences (Pillai–Bartlett statistic = 0.157; $F_{8,36} = 0.38$, p = 0.923) were observed amongst treatments in any of the main functional categories (with average abundance > 1%, see Suppl. material 1: fig. S10, table S7).

Discussion

Our study shows taxonomic and functional variation in soil microbial communities between invaded and uninvaded treatments once the invasive plant C. edulis was removed from the field experiment with a non-aggressive eradication method. Lack of strong differences in microbial communities seven months after removal contrasted with the ones observed in some chemical properties, such as carbon and nitrogen content or pH. Uncoupled changes between the biotic and abiotic components suggest that, while microbial community shifts usually coincide with changes in physicochemical properties of the soil (Dawkins et al. 2022), the magnitude of change in the microbial community may differ from the one of soil chemical properties once the invasive species is no longer present. A tendency for recovery to pre-invasion conditions has been previously suggested, based on the dynamics of enzymatic activities (Novoa et al. 2013), but, to our knowledge, there is little information about microbiome status once the invasive species has been removed. Species richness (i.e. number of ASVs) was similar amongst treatments, but differences were found in the taxonomic and functional composition of soil microbial communities. Remarkably, fungal and bacterial communities showed contrasting responses, with the invasive and control treatments showing little overlap in species composition of bacteria while, in the case of fungi, the largest difference occurred between the invasive treatment and the combined treatment of invasive species and climate change. This result suggests that, although some species of the genus Carpobrotus are expected to particularly favour fungal communities (Badalamenti et al. 2016), an enhancement of warm and drought conditions seemed to counteract such effect, which was probably driven by the increase in water holding capacity associated with the presence of this mat-forming succulent invader (Novoa et al. 2014; Badalamenti et al. 2016). This would explain why our results contrast with the ones reported by Anthony et al. (2020), who showed that the impact of garlic mustard invasion on soil fungi would be enhanced under warmer conditions. Taken altogether, our findings indicate that C. edulis could have a significant impact on belowground diversity, with potential effects on the soil microbiome, as documented here and in earlier research (Novoa et al. 2020; Rodríguez-Caballero et al. 2020; Caravaca et al. 2022). Nonetheless, it seems that some of these effects may be ameliorated under rising temperatures and drought conditions predicted by climate change.

Shifts in microbial species composition were not associated with changes in species richness and, remarkably, drove little differences in functional diversity, mostly in the case of bacteria. Aggregate metrics, such as species richness, may mask relevant diversity changes (Hillebrand et al. 2018). On the contrary, compositional turnover metrics may detect community shifts even in the absence of changes in the number of species (i.e. species loss). This is particularly important because soil microorganisms may not be active, but persist under adverse conditions, such as drought, and recover and regrow when conditions are favourable again (Jansson and Hofmockel 2020). Hence, community composition assessments based on relative abundance metrics are more appropriate to detect such changes. Nevertheless, caution is needed in hyperdiverse systems, such as microbial communities, as compositional turnover may detect community changes, but these may still have limited consequences for ecosystem functioning. In fact, our results suggest high functional redundancy within the microbial community of this coastal ecosystem and, as such, only small differences in functional diversity of fungi were observed amongst treatments.

Saprotrophic fungi were more abundant in treatments where C. edulis had been present. Previous studies have attributed similar results to the increase in dead organic matter (Caravaca et al. 2022), given that C. edulis invasion leads to higher plant biomass (Campoy et al. 2021) and saprophytic microbes will be driven primarily by the quality and quantity of organic materials. This result would be in agreement with previous meta-analyses reporting that plant invasions tend to increase litter quality and soil N availability (Liao et al. 2008). Notably, we did not observe an increase of plant pathogens associated with C. edulis. This result contrasts to some extent with the findings of Caravaca et al. (2022), who documented an enrichment of native pathotrophs, especially plant pathogens, in the fungal community of the C. edulis rhizosphere, an outcome expected to facilitate its invasion (Caravaca et al. 2022). In general terms, our results show little functional effects of C. edulis in soil microbiomes shortly after removal, which is consistent with the small differences observed in taxonomic diversity and, in general, with the limited impact on soil microbial diversity reported in this study. Nonetheless, these results are unavoidably biased by the Raunkiæran shortfall (sensu Hortal et al. (2015)) in our knowledge of biodiversity, as functional traits were only assigned to a fraction of taxa, implying that functional differences may exist amongst treatments, but we are not aware of them yet. Despite the major recent advances in the field of functional microbiology, this remains a barrier to our progress in comprehending ecosystem functioning and its response to global change.

While biotic differences were subtle amongst treatments, there were significant variations in soil chemistry between the invasive species treatment and the others, evidencing that some physicochemical changes may persist for a given time after the removal of the invasive species. These findings are consistent with the results of previous studies showing variations in pH, inorganic N and in the availability of several nutrients in previously invaded sites (Vilà et al. 2006; Molinari et al. 2007; Conser and Connor 2009; Novoa et al. 2013; Novoa et al. 2014; Badalamenti et al. 2016; Vieites-Blanco and González-Prieto 2018a) and provide further evidence of the high variability on the magnitude and direction of the effects of *C. edulis* depending on the region and habitat (Novoa et al. 2014; Vieites-Blanco and González-Prieto 2018a; Rodríguez-Caballero et al. 2020). In fact, such locality-specific responses may explain some of the differences observed here, although

the most striking one, i.e. the decrease in soil total N we found in the invasive treatment, could be also attributed to the relatively short duration of the experiment (i.e. 14 months), which would have prevented litter accumulation and decomposition. Most changes in soil chemistry are likely caused by leaching from *C. edulis* litter (Conser and Connor 2009; Vieites-Blanco and González-Prieto 2018a) but, if plants are removed before litter is accumulated, key nutrients and cations would be removed as well. For instance, *C. edulis* necromass has been shown to be richer in Mg and Ca than native necromass, leading to lower concentrations of these cations in the soil (Vieites-Blanco and González-Prieto 2018a). More remarkably, our findings indicate that warming and drought may mitigate changes in soil properties driven by the invasive species, as the combined and control treatments were highly similar in soil chemistry.

Invasive plants affect soil biota through litter and rhizosphere pathways (Zhang et al. 2019), but the magnitude of their impacts, including those of C. edulis, can greatly increase with the time since invasion (Corbin and D'Antonio 2012; Novoa et al. 2012). Thus, the small short-term effects observed on the soil microbiome following the removal of C. edulis could be attributed to the relatively short duration of the experiment, even though shorter experiments (i.e. 3-months) have been sufficient to alter Carpobrotus' soil microbiome in greenhouse experiments (de la Peña et al. 2010). This interpretation aligns with the idea that soil microbiota recovery is quicker when community shifts are associated with changes in relative abundances rather than the elimination of certain taxa (Corbin and D'Antonio 2012). Moreover, in well-drained and aerated dune soils, the concentration of allelopathic compounds should rapidly decrease and consequently their phytotoxicity (Bonanomi et al. 2006b). Besides, it is also well established that phytotoxic substances released during litter and organic matter decomposition may persist briefly in the soil, since they can be rapidly degraded by physical (temperature and light) and chemical processes as well as by soil microorganisms. Consequently, their inhibitory effects usually last only a few weeks and are limited to short-term phases of the early decomposition stages (e.g. Bonanomi et al. (2006a)). This could also explain why removal of invasive species in coastal ecosystems may reduce some of the effects on soil microbial communities (Parsons et al. 2020). In the case of C. edulis, Novoa et al. (2013) showed that many soil properties, as well as enzymatic activities, tend to recover to pre-invasion conditions after plant removal. Here, we also demonstrate that such recovery tendency may be accelerated in a climate change scenario. Therefore, although climate change is expected to increase growth and physiological performances of C. edulis, thus favouring its invasion (Campoy et al. 2021), the adoption of quick eradication measures may result in smaller soil microbiome shifts than at present climatic conditions.

Conclusion

Contrary to our initial prediction, here we show that climate change may reduce some of the short term effects of *C. edulis* on soil microbiome after removal from a coastal dune ecosystem. Nevertheless, our results also indicate that an invasion time of just 14 months is sufficient to result into differences on soil chemical properties (e.g. pH, EC and nutrient availability) and soil microbiome between invasive and control treatments seven months after plant removal. Given that effects on soil microbiome were relatively small, our findings support the low impact of commonly-used eradication measures, such as hand-pulling (Campoy et al. 2018). The removal of living biomass and litter by hand-pulling seems to be instrumental for soil microbiome preservation in sites previously invaded by *C. edulis*. This removal needs to eliminate both living parts and litter (Novoa et al. 2013), as well as prevent plant re-rooting (Núñez-González et al. 2021) and soil erosion (Chenot et al. 2018) to ensure the recovery of diverse native plant communities of coastal sites (Buisson et al. 2021). Our study is a step forward towards understanding potential interactions between plant invasions and climate change, revealing an unexpected complexity in the outcome of this interaction for belowground microbial communities. More field studies, including long-term surveys of restored ecosystems are needed and, ideally, should be integrated into the global framework for monitoring soil biodiversity and ecosystem function (Guerra et al. 2021).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

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Data availability

All of the data is available upon request to the corresponding author.

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Supplementary material 1

Supplementary information

Authors: Carola Gómez-Rodríguez, Rubén Retuerto, Josefina G. Campoy, Susana Rodríguez-Echeverría Data type: pdf

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Supplementary material 2

R code

Authors: Carola Gómez-Rodríguez, Rubén Retuerto, Josefina G. Campoy, Susana Rodríguez-Echeverría Data type: R file

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Research Article

Explosive spread of sand dropseed (*Sporobolus cryptandrus*), a C₄ perennial bunchgrass, threatens unique grasslands in Hungary (Central Europe)

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Abstract

Sporobolus cryptandrus is a C_4 perennial bunchgrass native to extensive areas of North America. As a non-native species, it has been reported from several continents, and it has been described as a transformer species in sand steppes of Central and Eastern Europe. However, its spreading ability across the landscape and within habitats has not been quantified, and factors determining its success have not yet been assessed.

In this study, we focused on the largest stronghold of *S. cryptandrus* invasion in Hungary, where the species was first recorded in 2016, and investigated its present distribution in the landscape by mapping along dirt roads. In a separate local study in a heavily infested sand dune site of 2 km², we assessed the infestation level and factors affecting the species' establishment.

Our landscape-scale mapping found that in April 2023, the distribution of *S. cryptandrus* encompassed a largely contiguous 600 km², with documented presence from 282 1-km² mapping units. The species occurred more than 5 m away from roads in 71 mapping units, mostly in the centre of its distribution area. *Sporobolus cryptandrus* presence was negatively related to soil organic matter content and positively related to sand content. At the local scale, we found the species in 39% of vegetation plots in a sand dune site originally covered by Pannonic sand steppes, a priority habitat in the EU Habitats Directive. *Sporobolus cryptandrus* presence at this site was negatively related to the total cover of resident grassland but, surprisingly, was unrelated to the distance from roads. Collectively, these results suggest that primary spread occurs mostly along roads; these roadside populations likely serve as sources for establishment in neighbouring grasslands, but subsequent mass invasion becomes independent from roads.

Together with the species' broad macroclimatic tolerance in North America and reported mass invasion events in Ukraine and Russia, our results suggest that *S. cryptandrus* likely poses a broad-scale threat to Eurasian dry grasslands, in particular on coarse-textured sandy soils with low vegetation cover. **Key words:** Dirt roads, distribution, invasive grass, mapping, open habitats, sand steppe, sandy soil, unproductive soil

Introduction

Invasive grasses pose serious threats to natural communities by substantially altering the biodiversity and functioning of ecosystems (D'Antonio and Vitousek 1992; Williams and Baruch 2000). On a global scale, the Poaceae family is highly represented among naturalised alien species, with greater proportion of perennial than annual grasses (Pyšek et al. 2017). Furthermore, about half of the invasive perennial grass species of the world have a C_4 photosynthetic pathway (Sage et al. 1999; Weber 2017), which may promote their expansion in the future when the frequency of episodic heat events, fire, and forest canopy opening are projected to increase (Sage and Kubien 2003). Nevertheless, introduced C_4 perennial bunchgrasses are relatively rare in the European flora (Weber 2017).

Sand dropseed (Sporobolus cryptandrus (Torr.) A. Gray) is a C₄ NAD-ME perennial bunchgrass (Koteyeva et al. 2023), which has a wide native distribution range in North America (southern Canada, the United States except for its south-eastern part, and northern Mexico; Holub and Jehlík 1987; Peterson et al. 2007; Kartesz 2015), and is also native to Argentina (Cavagnaro 1988; Rauber et al. 2020). The status of this species in its native range varies from endangered or threatened rare species in some north-eastern states of the USA (Connecticut Department of Energy and Environmental Protection 2015; New Hampshire Official Rare Plants List 2020) to a common species that can occur even along roadsides in the Intermountain Region (Kartesz 2015; Tilley et al. 2022). Sporobolus cryptandrus is typically associated with sandy soils and open habitats with bare soil surfaces (Ramaley 1939; Albertson and Weaver 1944; Hulett et al. 1966, 1988). This is a characteristic species of the vegetation in sand hill areas of the Great Plains (Saskatchewan, Nebraska, and Colorado), both as a pioneer species of rolling sand dunes and as a frequent component of sand grasslands on stabilised dunes and level uplands (Ramaley 1939; Hulett et al. 1966). In addition, sand dropseed may be one of the dominant grasses in the herbaceous layer associated with the shrub Artemisia filifolia in sandsage prairies in sand dune areas (Hulett et al. 1988). Sporobolus cryptandrus is a common species of short grass and mixed grass prairies in the central and southern Great Plains, but is usually abundant only on sandy soils and in areas denuded by drought (Albertson and Weaver 1944; Shiflet 1994). In Argentina, sand dropseed is usually a low-abundance species (< 5% cover) in pampa grasslands and in the grass layer of shrublands (Cavagnaro 1988; Rauber et al. 2020).

Outside of its native range, casual or naturalised populations of *S. cryptandrus* have been reported from numerous locations in Eurasia (from Spain to Japan), Australia and New Zealand (Suppl. material 1). Although *S. cryptandrus* has been detected primarily in disturbed habitats, such as roadsides, along dirt roads, overgrazed or formerly burnt grasslands, ploughed sandy areas, and mown urban grasslands, it can also successfully enter natural plant communities (Nobis et al. 2015; Maltsev et al. 2017; Demina et al. 2018; Török et al. 2021). Recent studies from Central and Eastern Europe, including Hungary, highlight that this grass has

become a transformer invasive species (Maltsev et al. 2017; Demina et al. 2018; Török et al. 2021; Hábenczyus et al. 2022). *Sporobolus cryptandrus* was found to be the only perennial grass among the neophyte species of Hungarian and Russian sand steppes originally dominated by perennial bunchgrasses (Csecserits et al. 2016; Maltsev et al. 2017). The high cover of *S. cryptandrus* in sand steppes was found to be associated with reduced species richness and abundance of subordinate species, homogenised vegetation composition, or even a complete replacement of the originally dominant native perennial grasses (Maltsev et al. 2017; Török et al. 2021; Hábenczyus et al. 2022). Therefore, a detailed assessment of the spreading ability and rate of this grass at the landscape and local scales, as well as the factors determining its invasion success, is urgently needed.

The overall objective of our study was to investigate the current distribution of *S. cryptandrus* at the species' largest naturalised population in Hungary and identify factors associated with its successful spread at the landscape and local scales. At the landscape scale, we mapped *S. cryptandrus* along dirt roads and neighbouring habitats and tested if the species' presence is related to soil characteristics and specific habitats. At the local scale, we assessed the species frequency, and tested if its occurrence is related to distance from roads and the total cover of resident grassland in a heavily infested site of Pannonic sand steppes, a priority habitat type of the EU Habitats Directive (Natura 2000 code: 6260*; Directive 2013) and a critically endangered habitat (Janssen et al. 2016).

Methods

Study region

In Hungary, *S. cryptandrus* was discovered in 2016, near the city of Kiskunhalas (Danube–Tisza Interfluve, Central Hungary) and in the city of Debrecen (eastern Hungary; Török et al. 2021). A historical record of the species was documented in 1927 from the city of Győr (north-western Hungary; Polgár 1933), but the data has not been confirmed since then. Currently, *S. cryptandrus* occurrences have been reported from three regions of Hungary: Danube–Tisza Interfluve (several populations between the city of Lajosmizse (Central Hungary) and the village of Ásotthalom (southern Hungary)), Northern Great Plain (in the city of Debrecen and close to the city of Létavértes), and Western Transdanubia (near the village of Mezőörs; Suppl. material 1).

The study was conducted in the southern part of the Danube–Tisza Interfluve, Central Hungary, where the largest number of *S. cryptandrus* occurrence data was recorded prior to our systematic mapping (Suppl. material 1). The climate of the region is temperate continental with a sub-Mediterranean influence. Mean annual temperature is 10.4 °C, and mean annual precipitation is 500–550 mm with a mid-summer drought typical in July and August (1961–1990; Kovács-Láng et al. 2000). Dominant soil types are coarse-textured calcareous sandy soils with high (> 90%) sand content and low (< 3%) humus content (Kovács-Láng et al. 2000; Kröel-Dulay et al. 2019). Major habitat types include agricultural fields, tree plantation forests of native and alien species, secondary grasslands on previous arable lands, and the remnants of natural Pannonic sand forest steppe mosaic of open and closed grasslands, and juniper-poplar shrublands (Fig. 1).



Figure 1. The setting of the study area in Central Europe and Hungary (red), and its habitat map. The red symbol on the habitat map indicates the local study site. The land cover information is based on the Ecosystem Map of Hungary (Tanács et al. 2022).

Landscape-scale mapping

Since we planned to map the current distribution of *S. cryptandrus*, we did not have an *a priori* defined study area. As a starting point, we used *S. cryptandrus* occurrence data from the database of the Kiskunság National Park and from the co-authors of this paper, in the vicinity of the first record of the species, south-east of the city of Kiskunhalas (Török et al. 2021). Note that these data were spatially aggregated and temporally uneven, thus not suitable for reconstructing the invasion dynamics of *S. cryptandrus* between 2016 and 2022.

We implemented the mapping by driving slowly (max 15 km/h) on dirt roads and visually searching for *S. cryptandrus* individuals along the roads. We decided to sample along dirt roads because (a) large populations of *S. cryptandrus* were reported to occur along dirt roads (Török et al. 2021), (b) the low speed allowed us to spot *S. cryptandrus* also in the neighbouring vegetation, and (c) this method made it possible to systematically sample a relatively large area within a reasonable timeframe. The mapping was carried out in early spring (between February and April) 2023. According to our experiences, the dry *S. cryptandrus* bunches and flowering stems from the previous summer are easily recognisable in the open vegetation along the dirt roads as well as in the neighbouring grasslands and forest understory at this time of the year (Fig. 2).

During mapping, a new *S. cryptandrus* occurrence was only recorded when we were at least 120 m (checked on GPS) from an already documented occurrence. We recorded both the presence along the road, defined as within 5 meters of the road edge, and the presence further away from the road, defined as more than 5 meters from the road edge. Based on our field experience, we think that we can,



Figure 2. Sporobolus cryptandrus in the study area, south-east of the city of Kiskunhalas. The species most frequently grows on and along dirt roads (upper left picture; 7 February, 2023), but it can also form monodominant patches away from roads (upper right picture; 7 February, 2023). It can establish and grow in natural grasslands (lower left picture; large green tussocks in the foreground are *S. cryptan-drus*, whereas small yellowish bunches are native *Festuca vaginata*, 13 September, 2022) and take over dominance (lower right picture; 13 September, 2022). Pictures were taken by G. Kröel-Dulay and A. Rigó.

with high probability, spot average-sized *S. cryptandrus* individuals to at least 30 m in open grasslands, where the species occurs. Starting in the close vicinity of the first reported occurrence (recorded in 2016) and moving gradually away, we sampled all dirt roads unless they were closer than 1 km to an already sampled parallel road. We gradually expanded the search area based on newly detected occurrences, until no additional occurrences were found within a ca. 2 km radius from the outermost occurrences. In the case of already known or accidentally found sporadic occurrences, we only searched their neighbourhood. Mapping in some areas was constrained by the lack of dirt roads or inaccessibility (e.g. fenced areas). While driving, we recorded not only *S. cryptandrus* occurrences, but also the tracks we drove, in order to report the verified absence of the species.

For the visualisation of *S. cryptandrus* distribution in the landscape scale, we plotted our data using the EEA reference grid cells of the ETRS89-LAEA Europe coordinate system (EPSG 3035; EEA 2013) with a 1-km resolution. We distinguished three grid cell states: (1) "presence", where *S. cryptandrus* was observed during our sampling or beforehand, (2) "verified absence", where we drove at least 500 m, but no *S. cryptandrus* was found during our sampling (or beforehand), and (3) "no data", where we drove less than 500 m and no *S. cryptandrus* was found during our sampling (or beforehand). Within the grid cells where *S. cryptandrus* was present, we identified cells where the species was recorded more than 5 m



Figure 3. Distribution of *Sporobolus cryptandrus* within the study area at a 1-km resolution in April 2023 and the first documented record in 2016. Verified absence denotes cells where at least 500 m dirt roads were sampled, and no *S. cryptandrus* was found, while no data denotes cells that were not sampled, or less than 500 m dirt roads were sampled and no *S. cryptandrus* was found. The core area was defined as the convex hull around the outermost occupied cells that are not separated from the large areas of mostly contiguous distribution (occupied cells) by more than one empty cell. *Sporobolus cryptandrus* presence further away from roads means cells where the species was present more than 5 m away from roads (in almost all of these cases, it was found also along the roads).

away from the road edge. We defined the core area of the current distribution (April 2023) as the convex hull around the outermost occupied cells that are not separated from the large areas of mostly contiguous distribution (occupied cells) by more than one empty cell (Fig. 3). We defined the core area for two reasons. First, we think that this area is a more robust measure of the state of invasion than the convex hull of all known occurrences, because it is less sensitive to one or a few data points. Second, we used *S. cryptandrus* occurrences within the core area for further analysis on preferences to soil and habitat types, because these relationships may not be visible in areas with extremely few occurrences. All maps were created using ESRI ArcMap 10.8 GIS software (ESRI 2020).

Local survey in a highly infested grassland site

We studied the local-scale distribution of *S. cryptandrus* in a heavily infested sand dune system close to the first record of the species within the study area (coordinates: 46.414, 19.550 EPSG 4326; Fig. 1) in July 2022. Please note that the peak of plant biomass production in sand grasslands is usually in June, thus sampling in July provided a reliable estimate of species abundances in this system. Within a ca. 2 km² area covered predominantly by Pannonic sand steppes, we selected 100

randomly located 16 m² plots for vegetation sampling. As we aimed to sample sand steppes, a randomly selected plot location was skipped, and a new plot location was generated, if it fell on a road, in a shrubby or forested patch, or closer than 50 m to a previously sampled plot. In each plot, we recorded the presence and estimated the percentage cover of *S. cryptandrus*, as well as all other vascular plants that reached at least 1% cover.

Statistical analysis

Statistical analyses were performed using R version 4.1.1 (R Core Team 2022). For statistical analysis on what environmental factors may be related to the present distribution at the landscape scale, we first generated absence data points within the core area by randomly placing points to the tracks that we drove using the Generate Points Along Lines tool of ESRI ArcMap 10.8. The spatial constraint was that a new absence point should be at least 120 m from a presence or an already chosen absence point. Finally, we ended up with a total of 5715 points, 873 presence and 4842 absence points within the core area. For each point, we gathered soil and habitat information as potential environmental factors that may be related to S. cryptandrus presence. Soil sand content (%) and soil organic matter (SOM) content (%) were derived from the DOSoReMI database (https://dosoremi.hu/ en/; Pásztor et al. 2020). For habitat information, we used the presence/absence of major habitat types (open grassland, closed grassland, cropland, coniferous forest, broad-leaved forest) within a 20-m radius around each point, derived from the Ecosystem Map of Hungary (Fig. 1; Tanács et al. 2022). This buffer zone was used because S. cryptandrus presence/absence localities were studied along dirt roads, which are often habitat boundaries, and we wanted to include only habitats in the close vicinity of each point.

We modelled the effect of habitat types, and the percentage sand and SOM contents of the soil on the presence of *S. cryptandrus* with three separate binomial generalized linear models. We used separate models because sand and SOM contents were correlated, and habitat types were also related to soil properties. For the three models, we first used all sampling points but found significant spatial autocorrelation in the residuals based on Moran's tests in the DHARMa R package (Hartig 2022). To avoid spatial autocorrelation, we selected a subset of 500 random points from the 5715 points, with a minimum distance of 500 m between them, and repeated the analyses on this subset. In the case of the subset models, we did not detect any spatial autocorrelation; therefore, we used these models to present the results.

In order to explain *S. cryptandrus*' presence within a highly infested grassland site in the local survey, we included the distance from the nearest dirt road and the total cover of resident grassland as explanatory variables in two separate binomial generalized linear models. The dirt road map was obtained by digitizing frequently used roads from a satellite image, and we used the Near tool in ESRI ArcMap 10.8 to get a distance measure for each plot (n = 100). The cover of resident grassland was obtained by summing up the percentage cover of each species present in the given plot (without *S. cryptandrus*). Plots with *S. cryptandrus* cover above 5% (15 plots) were left out of this analysis, because a substantial *S. cryptandrus* cover may itself induce a decrease in the cover of the resident species. We tested for but found no spatial autocorrelation in the model residuals by using the DHARMa

R package (Hartig 2022). All models were checked visually for and fulfilled the homoscedasticity assumption of the residuals. Results were visualised with the gg-plot2 package (Wickham 2011).

Results

We sampled (drove) a total of 1326 km and detected 902 *S. cryptandrus* occurrences (with the constraint that neighbouring occurrences are at least 120 m from each other), spread over 266 1-km² units of the EEA reference grid. After merging these with previous data collected between 2016 and 2022, *S. cryptandrus* occurred in 282 mapping units (red cells in Fig. 3). Its presently known distribution extends 46 km in an east-west and 45 km in a north-south direction. Within the 591 km² core area (Fig. 3), *S. cryptandrus* occurred in 268 mapping units (45%). The species occurred more than 5 m away from roads in 71 mapping units, mostly in the centre of its distribution (Fig. 3).

Across the core area of its present distribution in the study area, the probability of *S. cryptandrus* presence was negatively related to soil organic matter content $(\chi^2 = 43.71, df = 1, p < 0.001; Fig. 4a, Suppl. material 2)$ and positively related to sand content $(\chi^2 = 11.12, df = 1, p < 0.001; Fig. 4b)$. Furthermore, the probability of *S. cryptandrus* presence along dirt roads was positively related to the presence of open grasslands $(\chi^2 = 19.25, df = 1, p < 0.001)$ and coniferous forests $(\chi^2 = 24.00, df = 1, p < 0.001)$, negatively related to the presence of croplands $(\chi^2 = 17.09, df = 1, p < 0.001)$, and unrelated to the presence of closed grasslands $(\chi^2 = 0.21, df = 1, p = 0.649)$ and broad-leaved forests $(\chi^2 = 0.01, df = 1, p = 0.947; Fig. 4c)$.



Figure 4. The relationship between the probability of *Sporobolus cryptandrus* presence at the landscape scale and **a** soil organic matter (SOM) content **b** soil sand content, and **c** the presence or absence of major habitat types within a 20-m radius around the sampling points along dirt roads.



Figure 5. The modelled relationship between the probability of *Sporobolus cryptandrus* presence in plots within a heavily infested open grassland site and **a** the distance from the nearest dirt road (n = 100) and **b** the total cover of the resident grassland community (n = 85). To study the relationship with the total cover, we left out plots with the *S. cryptandrus* cover over 5%, because we wanted to avoid *S. cryptandrus* substantially affecting the resident community in our samples.

In the heavily infested Pannonic sand steppe site in the middle of *S. cryptandrus* distribution, the species was present in 39% of vegetation plots, and out of these, *S. cryptandrus* was the dominant species (i.e. species with the highest cover in a study plot) in nine vegetation plots. The probability of *S. cryptandrus* presence was not related to how far a plot was located from dirt roads ($\chi^2 = 1.07$, df = 1, *p* = 0.300; Fig. 5a, Suppl. material 2). By contrast, it was strongly negatively related to the total cover of resident grassland ($\chi^2 = 27.16$, df = 1, *p* < 0.001; Fig. 5b), ranging from 80% probability in grasslands of 20% cover to very low probability in grasslands of 50% cover or higher.

Discussion

Our systematic mapping showed that in April 2023, *S. cryptandrus* had a largely contiguous distribution in a 591 km² area, occurring in about half of the mapping units within this area. This is striking because the species was first recorded in the region in 2016 (Török et al. 2021), and there have been only sporadic reports since then (Suppl. material 1), which indicates a very high spreading rate. Although the exact date of the establishment of the species in the area is unknown, it most likely happened not long before the first discovery in 2016 (Török et al. 2021), given the large size of the species' tussocks (up to 1 m high with inflorescences; Fig. 2) and the high intensity of botanical and ecological field surveys in the region.

The fast spreading of *S. cryptandrus* may be explained by its prolific seed production (a single panicle can produce ten thousand seeds; Weaver and Hansen 1939), effective seed dispersal (very small caryopses can easily be carried by the wind, and the pericarp becomes mucilaginous when moist; Peterson et al. 2007), and a large soil seed bank (Török et al. 2024). In addition, the species' ability to tolerate a wide range of climatic conditions may also have contributed to its rapid expansion in this semiarid sand dune system. In its native range, *S. cryptandrus* is described as an extremely drought-tolerant species adapted to sites receiving 175–410 mm mean annual precipitation (Tilley et al. 2009), and it also occurs even in cool climates with mean annual temperatures as low as 1 °C (Hulett et al. 1966). The rate of spread we found for *S. cryptandrus* (ca. 100 km² y⁻¹ on average since its first record in 2016) is in the higher part of a broad range (0.006–333 km² y⁻¹) of long-distance dispersal rates reported in the literature for alien herbaceous perennial species in their invaded range, and about fourfold higher than that of another invasive C_4 perennial bunchgrass, *Eragrostis lehmanniana* in Arizona grasslands (24.6 km² y⁻¹; Pyšek and Hulme 2005).

We detected *S. cryptandrus* further away from roads in 26% of occurrences (in almost all of these cases, the species was found also along the roads), particularly in the centre of the core area, in the vicinity of the first record of the species, where dirt roads were highly infested with *S. cryptandrus*. This distribution pattern suggests that *S. cryptandrus* spreads primarily along dirt roads but has an ability to enter natural grasslands (Fig. 2). These results indicate that *S. cryptandrus* has a greater invasion potential than other invasive species of open sand grasslands in the region. The annual *Ambrosia artemisiifolia* and *Cenchrus incertus* are also associated with dirt roads, but rarely colonise the adjacent intact natural vegetation (Szigetvári 2002). The perennial *Asclepias syriaca* was reported to be most abundant in tree plantations and old fields (Csecserits et al. 2016; Kelemen et al. 2016), and can only rarely invade natural sand grasslands (Albert et al. 2014; Csecserits et al. 2016).

We found that the presence of *S. cryptandrus* is positively related to the sand content of the soil, which is in agreement with previous reports on the species. In fact, the common English name of *S. cryptandrus* is sand dropseed, which reflects its preference for sandy soils in its native range (Ramaley 1939; Albertson and Weaver 1944; Hulett et al. 1966, 1988). In addition, its introduced populations are also frequently found on sandy soils (Gugnacka-Fiedor and Adamska 2010; Nobis et al. 2015; Török et al. 2021), and mass invasion events, in particular, took place in sand dune systems as well as on sandy floodplain terraces and riverbanks (Gouz and Timoshenkova 2017; Maltsev et al. 2017; Demina et al. 2018; Maltsev and Sagalaev 2018; Török et al. 2021). The probability of *S. cryptandrus* occurrence was negatively related to soil organic matter content. Although this relationship was kind of expected since sandy soils usually have low SOM, the relationship for SOM content was much stronger than for sand content: no plot with SOM over 1.8% had *S. cryptandrus*. This suggests that SOM may have a strong predictive power when estimating the species' potential future distribution.

The relationships of *S. cryptandrus* occurrence with the presence of specific habitats were consistent with the correlations between the species' presence and soil characteristics. The probability of *S. cryptandrus* presence along dirt roads was positively related to the presence of open grasslands and coniferous forests, habitat types that typically appear on soils with high sand and low soil organic matter content in the region (Kröel-Dulay et al. 2019). By contrast, the probability of *S. cryptandrus* occurrence along roads was negatively associated with the presence of croplands, which usually have soils with relatively high SOM and low sand contents. This is nicely exemplified by the broad southwest-to-northeast belt across our study area that is dominated by croplands (Fig. 1) and has few *S. cryptandrus* occurrences (Fig. 3). Note that areas northwest of this less suitable belt and thus separated from the first record and potential source of invasion, have a more fragmented *S. cryptandrus* distribution, with fewer instances where the species occurs further away from roads. This broad-scale pattern suggests that unfavourable landscapes may somewhat slow down, but do not ultimately prevent the spread of *S. cryptandrus*.

Our local survey in a sand dune site showed that once established, *S. cryptandrus* can become very frequent (it was present in 39 out of 100 random vegetation plots)

in natural sand grasslands within about six years. Furthermore, these occurrences were not correlated with the distance from dirt roads but were associated with low (< 50%) cover of the resident grassland. These results suggest that when *S. cryptandrus* enters natural grasslands, its further spread may become independent from dirt roads. Moreover, *S. cryptandrus* has become even dominant in several stands (in nine vegetation plots) since its first record in 2016. This implies that, as a dominant species, *S. cryptandrus* likely has a strong impact on community structure and ecosystem functions. Previous studies in the region demonstrated that the high cover of *S. cryptandrus* was associated with reduced species richness, increased vegetation height, and decreased abundance of certain plant functional groups (e.g. other perennials and insect-pollinated species; Török et al. 2021; Hábenczyus et al. 2022). However, long-term monitoring studies that follow the process of invasion in permanent plots are needed to provide a detailed description of how *S. cryptan-drus* invasion affects natural sand grasslands; we have already started such studies.

Collectively, the results of our landscape- and local-scale study revealed that *S. cryptandrus* has an ability to spread very fast along dirt roads, and these roadside populations likely serve as sources for its establishment in neighbouring grasslands, where the invasion of this grass poses a serious threat primarily to open perennial grasslands on unproductive sandy soils. These grasslands, the Pannonic sand steppes, are a priority habitat type of the EU Habitats Directive (Natura 2000 code: 6260*; Directive 2013). They harbour a high number of species endemic to the Pannonian biogeographical region (Fekete et al. 2014; Riezing 2023). This habitat type is considered critically endangered in the European Red List of Habitats (Pannonian and Pontic sandy steppe, E1.1a; Janssen et al. 2016), and 98% of the area of Pannonic sand steppes in Hungary were already lost in the last 230 years (Biró et al. 2018). Their remaining stands are already among the vegetation types that are most heavily infested by alien plant species in the country (Botta-Dukát 2008), and *S. cryptandrus* now becomes an additional serious threat.

The preference of S. cryptandrus to open grasslands at this stage of its invasion may be related to the fact that bare soils can provide a more favourable environment for seed germination of this grass. Previous studies reported that both diurnal temperature fluctuations, which are larger in bare soil than in soil beneath a closed grassland canopy (Thompson et al. 1977), and the presence of light increased the germination percentage of S. cryptandrus seeds (Sabo et al. 1979). However, it does not necessarily mean that more closed grasslands will not be in danger in the future. Consistent with this possibility, previous studies, both in our study area (near the city of Kiskunhalas) and in southern Russia, reported that S. cryptandrus can enter sand steppe habitats where the total vegetation cover (without S. cryptandrus) exceeds 50% (Demina et al. 2018; Hábenczyus et al. 2022). Together with the species' high spreading rate and its ability to be a transformer invasive species reported from Russia, Ukraine, and Hungary (Gouz and Timoshenkova 2017; Maltsev et al. 2017; Demina et al. 2018; Török et al. 2021; Hábenczyus et al. 2022), our results suggest that S. cryptandrus likely poses a broad-scale threat to Central and Eastern European grasslands on sandy soils.

The rapid spread of *S. cryptandrus* and its potential risk for natural sand grasslands call for developing effective management strategies against the species. Given the large population size and area covered by the species in our study area, full eradication is unrealistic. Therefore, preventing *S. cryptandrus* from further spread along dirt roads and particularly from entering natural grasslands should be the priorities for action. Our results highlight the need for regular monitoring of dirt roads in actual infested and high-risk areas, and for blocking roads in sites where *S. cryptandrus* are present in high densities. Our map on the current distribution of *S. cryptandrus* may help to identify roads that should be carefully monitored for new infestations within and around the study area, and newly established and small populations should be eradicated. Regarding the natural grassland sites where *S. cryptandrus* already reached relatively high abundance, further studies are needed to assess how various habitat management practices (e.g. grazing, mowing, prescribed burning or their combination) affect the competition between *S. cryptandrus* and resident native plant species. Finally, if the mass invasion of *S. cryptandrus* cannot be stopped or at least slowed down, representative stands of unique Pannonic sand steppes should be designated, which are intensively surveyed and actively kept free of the species.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Data availability

Data that support the findings of this study are available from the authors upon reasonable request. R scripts used for data analysis are available in Suppl. material 2.

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Supplementary material 1

Global distribution of sand dropseed (*Sporobolus cryptandrus* (Torr.) A. Gray) as an alien plant species based on literature data

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- Data type: xlsx
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Supplementary material 2

R scripts and the results of binomial generalized linear models used to test the effect of explanatory variables on the presence of *Sporobolus cryptandrus*

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Research Article

Testing a trapping protocol for generic surveillance of woodboring beetles in heterogeneous landscapes

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Abstract

Baited traps are a basic component of both specific and generic surveillance programs targeting wood-boring beetles at risk of introduction to new habitats because of global trade. Among the numerous protocols developed over the years for generic surveillance of longhorn beetles, jewel beetles, and bark and ambrosia beetles is the simultaneous use of black multi-funnel traps set up in the understory and green multi-funnel traps set up in the canopy of forested areas surrounding ports and other entry points. These traps are commonly baited with multi-lure blends of pheromones and host volatiles. In this study, we tested this trapping protocol in areas surrounding eight entry points located in Europe and North America to determine: i) the relative performance of black-understory traps and green-canopy traps among the targeted taxa; and ii) whether the dissimilarity among communities of beetles collected by the understory vs. canopy traps was affected by taxon and amount of forest cover in the traps' surroundings. A total of 96,963 individuals belonging to 358 species of wood-boring beetles were collected, including 21 non-native species. Black-understory multi-funnel traps were generally more efficient than green-canopy multi-funnel traps for detecting longhorn beetles and bark and ambrosia beetles, whereas the opposite trend was observed for jewel beetles. Differences between beetle communities caught in black-understory and green-canopy traps were mainly attributed to differences in species richness in jewel beetles, while both differences in species richness and species turnover contributed to the dissimilarity between communities of longhorn beetles and bark and ambrosia beetle. The difference in the number of jewel beetle species caught by the two trapping methodologies decreased with increasing forest cover, whereas species turnover increased when moving from an urban-dominated to a forest-dominated landscape. Overall, these results suggest that the simultaneous use of both black-understory and green-canopy multi-funnel traps can be considered a very efficient approach for generic surveillance of longhorn beetles, jewel beetles and bark and ambrosia beetles in both urban-dominated and forest-dominated areas surrounding entry points.

Key words: Buprestidae, Cerambycidae, early-detection, exotic species, monitoring, Scolytinae

Introduction

The continuous increase in global trade in recent decades, combined with deliberate plant introductions in the past, has resulted in increasing number of non-native insects moved outside their native ranges (Seebens et al. 2017; Isitt et al. 2024). This trend is evident for wood-boring insects, especially bark and ambrosia beetles (Coleoptera: Scolytinae), longhorn beetles (Cerambycidae) and jewel beetles (Buprestidae) (Rassati et al. 2016; Ruzzier et al. 2023a) which can be accidentally transported within wood packaging materials, logs, processed wood, and live nursery stock (Meurisse et al. 2019; Fenn-Moltu et al. 2023). After introduction, wood-boring beetles may become invasive pests, with high economic, ecological, and social impacts in urban and natural forests (Aukema et al. 2011). The jewel beetle Agrilus planipennis and the longhorn beetle Anoplophora glabripennis, for example, are listed among the top 10 of all invasive species for post-invasion management costs and are estimated to be among the costliest non-native insects worldwide (Cuthbert et al. 2022). For these reasons, substantial investments globally have focused on mitigating the arrival and establishment rate of non-native wood-boring beetles through more efficient biosecurity measures (Nahrung et al. 2023), including tools and strategies for early-interception of non-native species at and around entry points.

Among the numerous tools developed for surveillance of wood-boring beetles (Poland and Rassati 2019), traps baited with attractive lures are part of the biosecurity systems of several countries around the world (Rassati et al. 2015a, 2015b; Carnegie et al. 2018, 2022; Rabaglia et al. 2019; Hoch et al. 2020; Allison et al. 2021; Holusa et al. 2023; Mas et al. 2023; Wardhaugh and Pawson 2023). The knowledge acquired over the years on the key factors influencing trap efficacy (Allison and Redak 2017; Dodds et al. 2024) and on the chemical ecology of hundreds wood-boring beetle species (e.g., Byers 2007; Millar and Hanks 2017; Ranger et al. 2021) has led to the definition of a set of trapping protocols tailored to the target taxa or objective of the surveillance program. In the case of generic surveillance aimed at intercepting as many non-native species as possible, the simultaneous use of black multi-funnel or intercept panel traps set up in the understory and green multi-funnel traps set up in the canopy, all baited with multi-lure blends, in areas surrounding entry points (e.g., Wong et al. 2012; Hanks et al. 2018; Fan et al. 2019; Rice et al. 2020; Roques et al. 2023), might be considered as a potentially efficient approach. Baited black traps set up in the understory are known to attract longhorn beetles and bark and ambrosia beetles commonly living in the lower forest strata (De Groot and Nott 2001; McIntosh et al. 2001; Dodds et al. 2010; Flaherty et al. 2019; Ulyshen and Sheehan 2019), while baited green multi-funnel traps set up in the canopy are known to catch jewel beetles well (Francese et al. 2011, 2013; Petrice and Haack 2015; Skvarla and Dowling 2017; Santoiemma et al. 2024) but also longhorn beetles and certain bark and ambrosia beetles living in mid and upper forest strata (Rassati et al. 2019; Marchioro et al. 2020). Nonetheless, this trapping protocol remains to be tested in surveillance programs targeting areas surrounding high-risk sites, such as international ports and airports or warehouses, which can be characterized by heterogeneous landscapes ranging from urban-dominated areas to forest-dominated areas.

In addition to testing overall efficacy, there is an urgent need to better understand whether the simultaneous use of baited black multi-funnel traps placed in the understory and baited green multi-funnel traps placed in the canopy is always necessary, irrespective of the characteristics of the landscape. Previous studies showed that the efficacy of a trapping methodology can be context-dependent (e.g., Bouget et al. 2009; Marchioro et al. 2020; Rassati et al. 2021) as it can be affected by a number of environmental variables (Dodds et al. 2024), including the amount of tree cover occurring around the trap (Schroeder 2013; Nunes et al. 2021). In areas surrounding high-risk sites, traps can be set up either on isolated trees that are present along streets or private gardens (i.e., in urban-dominated areas) or on trees that are present in urban parks or forest patches and that are surrounded by many potential hosts (i.e., in forest dominated landscapes or where urban areas are intermixed with forest areas) (Bashford 2008; Rassati et al. 2015a; Mas et al. 2023). Thus, understanding whether the proposed trapping protocol can be simplified depending on the amount of tree cover occurring in the trap surroundings is crucial to optimize efforts and reduce overall costs of the surveillance program (Epanchin-Niell et al. 2014; Nguyen et al. 2024).

In this study, we conducted a trapping experiment in areas surrounding eight entry points located in Europe and North America using black multi-funnel traps set up in the understory and green multi-funnel traps set up in the canopy, all baited with the same multi-component blend of longhorn beetle pheromones complemented with plant volatiles. We first compared the relative efficacy of black-understory traps and green-canopy traps for detecting different target taxa, i.e., longhorn beetles, jewel beetles and bark and ambrosia beetles. Second, we calculated dissimilarity indices to compare the communities of longhorn beetles, jewel beetles and bark and ambrosia beetles collected by black-understory *vs.* green-canopy traps, and then we tested the effect of the amount of forest cover in the trap surroundings on the dissimilarity indices. These analyses allowed us to investigate whether the simultaneous use of black-understory traps and green-canopy traps is required irrespective of the taxon and the landscape in which this protocol is used, or whether a simpler protocol (e.g., using only black-understory traps) may detect as many species of a particular taxon, depending on the surrounding landscape.

Methods

Study sites, trap types and experimental scheme

The study was conducted at eight sites in five different countries in the temperate zone of Europe and North America: France, Italy, Switzerland, Canada (Nova Scotia) and USA (Ohio) (Suppl. material 1: fig. S1, table S1). Selected sites were characterized by the presence of mixed forest and urban patches, and were located in the proximity of entry points or high-risk areas such as ports, airports, warehouses and high-use recreation areas (Suppl. material 1: table S1).

At each site we used sixteen black and sixteen green multi-funnel traps (Suppl. material 1: fig. S1), supplied by Synergy Semiochemical Corporation (Delta, BC, Canada) and ChemTica Internacional (Santo Domingo, Costa Rica). Both trap types were pre-treated with a 50% solution of Fluon (active ingredient polytetrafluoroethylene) as a trap coating because of its ability to increase trap catches of wood-boring beetles (Graham and Poland 2012; Allison et al. 2016). Trap-collecting cups were filled with 150–200 ml of a 50% propylene glycol solution mixed with either water or as a pre-mixed marine/RV antifreeze solution. Propylene glycol solutions were replaced at each trap check.

Traps were set up using a $2 \text{ km} \times 2 \text{ km}$ grid as reference (Suppl. material 1: fig. S1), with one black and one green multi-funnel trap set up in each of 16 grid cells of $0.5 \text{ km} \times 0.5 \text{ km}$. The only exceptions were the two sites in France, where traps were coupled along a horizontal transect due to limits in space within the selected sites and local restrictions. Black multi-funnel traps were set up with the top of the trap about 1.5–2 m above the ground on lower tree branches, whereas green multi-funnel traps were set up in the upper one-third of the tree canopy at heights ranging from 7 m to 15 m. We selected this approach even though it confounded the effects of trap height and trap color on catch of targeted taxa because the latter was already addressed in several previous studies (reviewed in Dodds et al. 2024). Canopy traps were set up following the methods of Hughes et al. (2014). The two traps within the same grid cell were suspended on different trees, separated by 50-100 m. Trees were selected based on position and suitability to hold the weight of the traps, irrespective of the species. Traps were set up in mid-May 2019 and emptied every 2-3 weeks until the end of August 2019. All trapped longhorn beetles, jewel beetles, and bark and ambrosia beetles were identified to species level using morphological features and taxonomic keys. Voucher specimens were deposited in the insect collection of each institution.

Lures

All traps were baited with a lure containing a blend of eight cerambycid pheromones attractive to a wide range of longhorn beetle species (Fan et al. 2019; Roques et al. 2023), plus UHR (ultra-high release rate) ethanol and alpha-pinene lures. The pheromone blend included racemic fuscumol (volume amount: 50 mg), racemic fuscumol acetate (50 mg), geranyl acetone (25 mg), racemic 3-hydroxyhexan-2-one (50 mg), prionic acid (1 mg), 2-methylbutan-1-ol (50 mg), anti-2.3-hexanediol (50 mg), and monochamol (50 mg), all dissolved in isopropanol as a carrier to a total volume of 1 ml per lure (see Fan et al. 2019 for release rate). All pheromones were purchased from ChemTica Internacional (Santo Domingo, Costa Rica) except prionic acid (Alpha Scents Inc., West Linn, Oregon, USA); the ethanol and alpha-pinene lures were provided by Econex (Spain). One-milliliter aliquots of the pheromone blend were filled in glass vials with screw caps and stored at 4 °C until used. At the beginning of the trial and during each trap check, the 1-ml aliquots were poured into a clear polyethylene sachet containing a cotton cylinder, which was hung on the trap using a string. The addition of the two host volatile lures increases both the attractiveness of the pheromones to many species of longhorn beetles (e.g., Collignon et al. 2016; Miller et al. 2017) as well as the likelihood of trapping certain species of jewel beetles (Miller et al. 2015) and bark and ambrosia beetles (Miller and Rabaglia 2009; Marchioro et al. 2020).

Analysis of dissimilarity indices to investigate wood-boring beetle communities

To investigate differences in the communities of wood-boring beetles collected in black-understory multi-funnel traps and green-canopy multi-funnel traps, we used the β -diversity approach outlined in Carvalho et al. (2012) (see also Podani and Schmera 2011; Legendre 2014). The general term " β -diversity" refers to the total

compositional change between two communities (in our case study, the community of wood-boring beetles collected using the two trapping methodologies), and can be partitioned into two components: species richness difference and species replacement (Carvalho et al. 2012). "Species richness difference" refers to the relative difference in the number of species between two communities, whereas "species replacement" refers to the substitution of species by others when two communities are compared. The two components are additive, and their sum provides the β -diversity index.

Given "a" = number of species exclusive to the first community, "b" = number of species exclusive to the second community, and "c" = number of species common to both communities, the β -diversity is given by the Jaccard dissimilarity index:

$$\beta_{cc} = (a + b)/(a + b + c)$$

with values ranging from 0 (perfect similarity) to 1 (total dissimilarity). The species richness difference component is given by:

$$\beta_{\rm rich} = |a + b|/(a + b + c)$$

with values ranging from 0 (no richness difference) to 1 (maximum richness difference). The species replacement component is given by:

$$\beta_{-3} = 2 \min(a, b)/(a + b + c)$$

with values ranging from 0 (no replacement) to 1 (maximum replacement). To calculate these indices, data collected across the entire sampling season from black-understory and green-canopy traps (i.e., total catch per trap over the entire trapping season) of each grid cell at each site were paired, creating $n \times m$ presence/absence matrices for each taxon, whereby n = 2 (one row for each trapping method) and m = number of species. Then, for each matrix, the three indices were computed using the "vegan" package (Oksanen et al. 2022) in R software (R Core Team 2021).

Quantification of tree cover in the area surrounding the traps

Forest patches around each trap were manually digitized by visual inspection of high-resolution satellite images in Google Earth Pro (Google Inc.© 2023) within a buffer of 250 m radius. This spatial scale was selected based on the results of previous studies testing the attraction range of baited traps towards wood-boring beetles (e.g., Dodds and Ross 2002; Jactel et al. 2019). For each buffer, the total forest cover (%) was then quantified in GIS (Quantum GIS 3.22, QGIS Development Team 2021) after importing the digitized patches from Google Earth Pro (Google Inc.© 2023). Forest cover within the buffers ranged from 0% (where only urban patches were present) to 100% (where only forest was present), with a mean value (\pm standard deviation) of 51.4 \pm 39.3%.

Statistical analyses

We used generalized linear mixed models for all the analyses. Data collected from all sites were analyzed together to increase both the statistical power of the models and the gradient of forest cover around the traps. First, we tested the effect of the taxon and the trapping methodology on species richness and abundance. Species richness (i.e., total number of species) and abundance (i.e., total number of individuals) for each trap and pooled over the sampling rounds were considered as response variables. The taxon (categorical variable: longhorn beetles, jewel beetles, and bark and ambrosia beetles), the trapping methodology (categorical variable: black-understory and green-canopy multi-funnel traps) and their interaction were considered as explanatory variables. The site identity, the identity of each grid cell within each site and the identity of each trap within each grid cell were included in the models as nested random factors.

Second, we tested the effect of the taxon and the forest cover on beta-diversity indices. The three beta-diversity indices β_{cc} , β_{rich} and β_{-3} calculated for each pair of traps (within a cell) were considered as response variables. The taxon (categorical variable: longhorn beetles, jewel beetles, and bark and ambrosia beetles), the forest cover (continuous variable: mean % of forest cover in the buffers of 250 m radius around the pair of traps present in the same cell) and their interaction were considered as explanatory variables. The site identity and the identity of each cell of the grid within each site were included in the models as nested random factors.

Models were fitted with a Poisson distribution (log link function) for species richness, negative binomial distribution (log link function) for abundance, and Gaussian distribution for the beta-diversity indices. Pairwise comparisons between each taxon and between the two trapping methodologies within each taxon were run using Tukey correction of *p*-values. All the analyses were carried out in R software (R Core Team 2021). Models were fitted using the 'glmmTMB' (Brooks et al. 2022) and 'nlme' (Pinheiro et al. 2021) packages, and validated using the 'DHARMa' (Hartig 2022) and 'car' (Fox and Weisberg 2019) packages. There was no evidence of spatial autocorrelation of models' residuals, checked with Moran's I test for distance-based autocorrelation using the 'DHARMa' package (Hartig 2022). Pairwise comparisons and slope estimates were calculated using the 'emmeans' package (Lenth 2021).

Results

General results

A total of 96,963 individuals belonging to 358 species of wood-boring beetles were collected (Suppl. material 1: table S2). The most species-rich taxon was represented by longhorn beetles (169 species), followed by bark and ambrosia beetles (123) and jewel beetles (66). Longhorn beetles and bark and ambrosia beetles represented the most trapped species at all sites (Fig. 1). For abundance, bark and ambrosia beetles had the highest number of trapped specimens (73,109), followed by longhorn beetles (17,970) and jewel beetles (5,884) (Suppl. material 1: table S2). *Xyleborinus saxesenii* (48,042 individuals) and *Orthotomicus erosus* (4,180) were the most abundant ambrosia and bark beetle species collected, respectively, whereas *Phymatodes amoenus* (2,954) and *Agrilus olivicolor* (2,213) were the most abundant longhorn beetle and jewel beetle species, respectively. Although most of the trapped species were native, 21 species were non-native for 14,772 specimens. These non-native species included four longhorn beetle species (i.e., *Phymatodes testaceus* in North America, *Neoclytus acuminatus, Xylotrechus chinensis* and

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Figure 1. World map describing the communities of longhorn beetles, jewel beetles, and bark and ambrosia beetles collected at each experimental site. Circle size indicates the number of trapped species ranging from 55 (smallest circle) to 100 (biggest circle). The different colors within each circle indicate the relative percentage of species attributed to each taxon: red = longhorn beetles; green = jewel beetles; blue = bark and ambrosia beetles. Numbers in yellow circles represent the different study sites according to Suppl. material 1: table S1 column ID number.

Xylotrechus stebbingi in Europe), two jewel beetle species (i.e., *Agrilus cyanescens* and *Agrilus planipennis* in North America), and 15 bark and ambrosia beetle species (nine in North America, two in Europe and four in both continents) (Suppl. material 1: table S2).

Effect of taxon and trapping methodology on trap captures

Species richness was significantly affected by taxon and by the interaction between taxon and trapping methodology (Table 1). The mean number of species (\pm standard error) was significantly higher for longhorn beetles (8.34 ± 0.26) than bark and ambrosia beetles (7.42 ± 0.26) and jewel beetles (1.79 ± 0.15). In addition, the effect of trapping methodology differed depending on the taxon (Fig. 2A–C). For longhorn beetles, the mean number of species caught in black-understory traps was not significantly different from the number of species caught in green-canopy traps (Fig. 2A). For jewel beetles, green-canopy traps caught significantly more species than black-understory traps (Fig. 2B), whereas the opposite trend was observed for bark and ambrosia beetles (Fig. 2C).

Abundance was significantly affected by all tested variables (Table 1). The mean number of individuals (\pm standard error) was significantly higher for bark and ambrosia beetles (286.70 \pm 25.47) than for both longhorn beetles (70.47 \pm 4.03) and jewel beetles (23.08 \pm 5.43). In addition, black-understory traps generally caught more individuals than green-canopy traps, even though the trapping performance

changed depending on the taxon (Fig. 2D–F). Black-understory traps outperformed green-canopy traps for both longhorn beetles (Fig. 2D) and bark and ambrosia beetles (Fig. 2F), whereas the opposite trend was observed for jewel beetles (Fig. 2E).

Table 1. Analysis of deviance table from the generalized linear mixed models testing the effects of taxon (longhorn beetles, jewel beetles, and bark and ambrosia beetles), trapping methodology (black-understory multi-funnel traps and green-canopy multi-funnel traps) and their interaction on species richness (Poisson distribution; log link function) and abundance (negative binomial distribution; log link function). Nested random structure used for both models: ~1|Site/Cell/Trap. Type II Wald chi-square tests (χ^2), degrees of freedom (df), *p*-values, and lognormal marginal (mR²) and conditional (cR²) pseudo R-squared are provided for both models.

	χ^2	df	<i>p</i> -value
Species richness			
Taxon	659.517	2	< 0.001
Methodology	0.455	1	0.500
Taxon × Methodology	161.154	2	< 0.001
			$mR^2 = 0.73, cR^2 = 0.87$
Abundance			
Taxon	1040.747	2	< 0.001
Methodology	20.737	1	< 0.001
Taxon × Methodology	274.916	2	< 0.001
· · · · · ·			$mR^2 = 0.73$ $cR^2 = 0.86$



Figure 2. Mean (\pm standard error) species richness and abundance of longhorn beetles (**A**, **D**), jewel beetles (**B**, **E**), and bark and ambrosia beetles (**C**, **F**) for each trapping methodology. Asterisks within the plots indicate the statistical significance level from pairwise comparisons between the two trapping methodologies within each taxon from the generalized linear mixed models. Asterisks under the plots indicate the statistical significance level from pairwise comparisons among the three taxa from the generalized linear mixed models. *P*-values: * = 0.01 - 0.05; ** = 0.001 - 0.01; *** = < 0.001; ns = not significant (> 0.05). *P*-values were adjusted by Tukey correction. Model details are provided in Table 1.

Effect of taxon and forest cover in the trap surroundings on dissimilarity indices

 β_{cc} and β_{rich} were significantly affected by taxon and forest cover but not by their interaction, while $\beta_{.3}$ was significantly affected by taxon and by the interaction between taxon and forest cover (Table 2). Irrespective of the forest cover effect, jewel beetles showed higher β_{cc} and β_{rich} values, and lower $\beta_{.3}$ values compared to longhorn beetles and bark and ambrosia beetles (Fig. 3; Suppl. material 1: table S3). For all taxa, β_{cc} slightly decreased with increasing amount of forest cover,



Figure 3. Effect of forest cover in a buffer of 250 m radius around the traps on the dissimilarity among wood-boring beetle communities found in black understory multi-funnel traps and green canopy multi-funnel traps, considering the total beta-diversity β_{cc} (A–C) and its components species richness difference β_{rich} (D–F) and species replacement β_{-3} (G–I). Plots include model estimate (colored line) and 95% confidence intervals (colored shading). Model details are provided in Table 2. Pairwise comparisons among taxa are provided in Suppl. material 1: table S3. Slope estimates, along with their corresponding 95% confidence intervals, are provided in Suppl. material 1: table S4.

Table 2. Analysis of deviance table from the generalized linear mixed models testing the effects of taxon (longhorn beetles, jewel beetles, and bark and ambrosia beetles), forest cover (mean % in buffers of 250 m radius around the pair of traps) and their interaction on β -diversity (β_{cc}), species richness difference (β_{rich}) and species replacement ($\beta_{.3}$) indices (Gaussian distribution used for all models). Nested random structure used for all models: ~1|Site/Cell. Type II Wald chi-square tests (χ^2), degrees of freedom (df), *p*-values, and delta marginal (mR²) and conditional (cR²) pseudo R-squared are provided for all models. Pairwise comparisons among taxa are provided in Suppl. material 1: table S3. Slope estimates, representing the relationship between each index and forest cover, along with their corresponding 95% confidence intervals, are provided in Suppl. material 1: table S4.

	χ ²	df	<i>p</i> -value	
β_{cc}				
Taxon	391.167	2	< 0.001	
Forest	4.282	1	0.039	
Taxon × Forest	0.899	2	0.638	
$mR^2 = 0.53$, $cR^2 = 0.58$				
$\beta_{\rm rich}$				
Taxon	262.048	2	< 0.001	
Forest	4.617	1	0.032	
Taxon × Forest	5.073	2	0.079	
$mR^2 = 0.45, cR^2 = 0.47$				
β.3				
Taxon	17.470	2	< 0.001	
Forest	0.426	1	0.514	
Taxon × Forest	7.250	2	0.027	
$mR^2 = 0.07$, $cR^2 = 0.10$				

although the trend was not significant (Fig. 3A–C; Suppl. material 1: table S4). For jewel beetles, β_{rich} decreased with increasing amount of forest cover (Fig. 3E; Suppl. material 1: table S4), whereas the opposite trend was found for $\beta_{.3}$ (Fig. 3H; Suppl. material 1: table S4). For longhorn beetles and bark and ambrosia beetles, both β_{rich} and $\beta_{.3}$ showed no relationship with forest cover (Fig. 3D, F, G, I, Suppl. material 1: table S4).

Discussion

Our study confirmed that the use of baited traps around high-risk sites represents an efficient approach for generic surveillance of wood-boring beetles (Brockerhoff et al. 2006; Rassati et al. 2015a, 2015b; Fan et al. 2019; Rabaglia et al. 2019; Mas et al. 2023). The simultaneous use of black-understory multi-funnel traps and green-canopy multi-funnel traps baited with a multi-lure blend of pheromones and host volatiles allowed us to catch 21 non-native species from all the three targeted families (i.e., longhorn beetles, bark and ambrosia beetles and jewel beetles), as well as more than 300 native species. In addition to the importance of records of newly introduced or expanding non-native species, knowledge of distribution and abundance data for native species in areas near entry/export sites represents a second key benefit of the application of this or similar trapping protocols (Rassati et al. 2015a, 2018; Mas et al. 2023). In fact, these data can be crucial not only to increase our ability to predict which species are at most risk from being introduced in other countries via exported commodities (Mas et al. 2023) but also to monitor range expansion or shifts of native species, which could become invasive within their native distributional range (Rassati et al. 2018; Ruzzier et al. 2023b).

Comparing the efficacy of the two trapping methodologies, we found that black multi-funnel traps baited with the multi-lure blend and set up in the understory caught significantly more bark and ambrosia beetle species and individuals than green multi-funnel traps baited with the same blend and set up in the canopy, but significantly less jewel beetle species and individuals. For longhorn beetles, a difference between the two trapping methodologies was found in the total number of individuals (more in black-understory traps), but not in the number of species. The trends observed in our study are likely explained by the combined effect of trap height and trap color (Dodds et al. 2024), two variables that are well known to affect trap efficacy towards the targeted taxa. Several studies, for example, showed an increasing abundance and richness of longhorn beetles and jewel beetles with increasing trap height (Ulyshen and Hanula 2007; Maguire et al. 2014; Flaherty et al. 2019; Rassati et al. 2019; Sheehan et al. 2019; Sweeney et al. 2020) and/ or the opposite trend for bark and ambrosia beetles (Ulyshen and Hanula 2007; Hanula et al. 2011; Dodds 2014; Hardersen et al. 2014; Flaherty et al. 2019). Other studies showed that green colored traps are more efficient than black or dark colored traps in catching jewel beetles, especially Agrilus spp. (e.g., Crook et al. 2009, 2014; Francese et al. 2010a, b, 2011; Petrice and Haack 2015; Skvarla and Dowling 2017; Rassati et al. 2019; Tobin et al. 2021), and/or that black or dark colored traps are generally more or similar efficient than green colored traps in attracting bark and ambrosia beetles (Cavaletto et al. 2020; Marchioro et al. 2020) and longhorn beetles (Kerr et al. 2017; Rassati et al. 2019; Cavaletto et al. 2021). Although our study does not allow us to disentangle the individual contribution of trap color and trap height on beetle catches, it shows that the simultaneous use of black-understory and green-canopy multi-funnel traps in generic trapping programs targeting these three taxa at the same time is essential.

Analyzing dissimilarity indices, we also found that differences between beetle communities caught in black-understory traps and green-canopy traps were more evident for jewel beetles than for both longhorn beetles and bark and ambrosia beetles. For jewel beetles, these differences were mainly attributed to differences in species richness, while both differences in species richness and species turnover contributed to explain the dissimilarity of communities of longhorn beetles and bark and ambrosia beetles between trapping methodologies. These results are especially useful when planning surveillance activities targeting only one of the three taxa. For longhorn beetles and bark and ambrosia beetles, the simultaneous use of black-understory and green-canopy multi-funnel traps is always recommended, as different species with different flight patterns can be caught by these two trapping methodologies. Previous studies testing different trap types, environmental gradients, and/or lures (Skvarla and Dowling 2017; Flaherty et al. 2019; Rassati et al. 2019) confirmed that the diversification of trapping methodologies is always advantageous when targeting longhorn beetles and bark and ambrosia beetles (Dodds et al. 2024). For jewel beetles, especially Agrilus spp., green-canopy multi-funnel traps should be prioritized over black-understory traps in some, but not all, landscapes. In fact, jewel beetles were the only taxon for which dissimilarity indices were affected by the amount of forest cover in the trap surroundings. In particular, the difference in the number of jewel beetle species caught in black-understory and

green-canopy multi-funnel traps decreased with increasing amount of forest cover, whereas the species turnover increased. In other words, jewel beetle species collected in black-understory traps were a subset of the species collected in green-canopy traps in urban-dominated landscapes, whereas the number of jewel beetle species exclusive to either black-understory or green-canopy traps increased in forest-dominated landscapes. Thus, the use of black-understory along with green-canopy multi-funnel traps is recommended only in forest-dominated landscapes.

Conclusions

Baited traps are an essential component of both specific and generic surveillance programs around the world, making the development of efficient trapping protocols a research priority. Here we showed that the simultaneous use of black-understory and green-canopy multi-funnel traps baited with a multi-lure blend of longhorn beetle pheromones and host volatiles can be considered a very efficient approach for generic surveillance of longhorn beetles, jewel beetles and bark and ambrosia beetles in both urban-dominated and forest-dominated areas surrounding entry points. The only case in which this protocol can be simplified using only green-canopy multi-funnel traps is when targeting jewel beetles in urban-dominated landscapes. Despite the general efficiency of the trapping protocol we tested, it is very likely that not all longhorn beetle, bark and ambrosia beetle and jewel beetle fauna present in the sampled area was represented by trap catches. Fan et al. (2019), for example, using the same multi-lure blend, collected 48% of the 238 longhorn beetle species native to France, percentage that would be likely lower for bark and ambrosia beetles and jewel beetles. Overall, these results highlight that further improvements to the trapping protocol are possible. For example, the use of traps of different colors (e.g., yellow, blue) (Cavaletto et al. 2020, 2021) or traps integrated with more complex visual stimuli (Masaguè et al. 2024) is very likely to increase the diversity of species that can be collected, especially within those taxa strongly relying on color vision at the adult stage (e.g., Lepturinae). Similarly, advances in the knowledge of the chemical ecology of still understudied taxa (i.e., jewel beetles) will further improve trap attractiveness with direct benefits for national and international surveillance.

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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

GS: data curation, formal analysis, writing – original draft, writing – review & editing; AB: funding acquisition, writing – review & editing; CC: lure preparation, methodology; GC: beetle identification; MF: beetle identification, writing – review & editing; NF: investigation; JAF: funding acquisition, investigation, writing – review & editing; EKLF: beetle identification, investigation; FG: beetle identification; MMG: funding acquisition, investigation, writing – review & editing; CK: beetle identification, investigation; MM: conceptualization, investigation; DN: conceptualization, data curation, formal analysis; AMR: funding acquisition, investigation, writing – review & editing; JS: funding acquisition, investigation, writing – review & editing; KVR: beetle identification, investigation, writing – review & editing; AR: investigation, investigation; DR: conceptualization, data curation, project administration, validation, visualization, writing – original draft, writing – review & editing. All authors approved 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

Testing a trapping protocol for generic surveillance of wood-boring beetles in heterogeneous landscapes

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Data type: docx

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Research Article

The Japanese climbing fern (*Lygodium japonicum*) invasion in the U.S.; insights from chloroplast genome sequencing

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Abstract

Japanese climbing fern (*Lygodium japonicum*) is a vine native to the open forests of eastern Asia that has become an invasive species in the U.S. Herbarium records first noted this species in the U.S. in 1903 (Georgia), with spread to eight states by the end of the 1930s and current establishment in 10 states of the southeastern U.S. We aimed to ask three questions regarding the introduction of *L. japonicum*: (1) Was there a single Japanese climbing fern introduction or were there multiple introductions? (2) What is the distribution of genotypes in the U.S.? and (3) What are the source population(s) from the native range in Asia? We sequenced the chloroplast genome from 74 *L. japonicum* herbarium specimens representing 24 native and 50 invasive range populations. Seventeen haplotypes were found in the native range compared to three in the invasive range. Our results indicate *L. japonicum* has low genotypic diversity in the invasive range relative to the native range. Even with low genotypic diversity, these data suggest at least three introductions of *L. japonicum*. However, we were unable to define the native source population(s) of invasive *L. japonicum*.

Key words: Herbarium specimen, invasive species, multiple introductions, southeastern U.S., whole chloroplast genome sequencing

Introduction

The process of invasion has the potential to result in reduced genetic variation in introduced populations. If a single or small number of introductions leads to reduced variation, introduced populations may suffer from inbreeding depression and a lack of adaptive potential (Sakai et al. 2001, but see Dlugosch et al. 2015). Most non-native plant species are introduced intentionally for agricultural or aesthetic purposes or unintentionally as contaminants (Mack and Erneberg 2002). Both vectors are expected to be recurrent, which leads to a high probability of multiple introductions. Indeed, single introductions of a plant invasive species are less common than multiple introductions (Dlugosch and Parker 2008). As the number of introductions increases, and admixture occurs between different lineages, novel multi-locus genotypes and increased heterozygosity could increase mean fitness of the invasive (Lee 2002; Colautti and Lau 2015-but see Barker et al. 2019). Uncovering the origin, number of introductions, and genetic diversity of invasive species is important for understanding their history and for their ultimate control (Sakai et al. 2001; Ward et al. 2008). In this study we aim to establish the



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Copyright: [©] Morgan L. Markley et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). number of introductions, source area, and geographic distribution of genotypes in an invasive fern species.

Japanese climbing fern [Lygodium japonicum (Thunb.) Sw.] is a vine native to the open forests of East Asia, South Asia, and portions of the East Indies (Hanks 1998). In the U.S. L. japonicum has become an invasive species, expanding rapidly in the 20th century (Van Loan 2006). Herbarium records first note this species in the U.S. in 1903 (Georgia), with spread to eight states by the end of the 1930s and current establishment in 10 states of the southeastern U.S. (SERNEC Data Portal. 2023). This species is found growing in forests, along waterways, and in disturbed roadside ditches. Japanese climbing fern has twining fronds of indeterminant growth- petioles continue to elongate indefinitely and can reach 90 feet in length (Minogue et al. 2009). This growth pattern allows for a dense canopy that shades out underlying native vegetation. Many reproductive life history characteristics of non-native plants have been proposed to facilitate greater competitive ability and eventual invasion. Such characteristics include self-fertilization, rapid growth to reproductive age, high and continuous seed/spore production, and adaptions for dispersal (Baker 1965). Lygodium japonicum possesses a number of these characteristics, combined with a reproductive strategy that presumably allows for rapid geographic spread. Lott et al. (2003) found that more than 90% of isolated L. japonicum gametophytes produced successful sporophytes via gametophytic selfing. Lygodium japonicum sporangia each produce 256 spores (Murtaza et al. 2004), and even a single spore could suffice to found a new Japanese climbing fern population. Due to this reproductive potential and its ability to reach above tree canopies, long distance wind dispersal and colonization of L. japonicum may be achieved by the successful establishment of single spores.

While considerable research exists on the invasion dynamics and control of the congener Old World climbing fern [Lygodium microphyllum (Cav.) R. Br.] (Volin et al. 2004, 2010; Gandiaga et al. 2009; Humphreys et al. 2017; David et al. 2020) relatively few focus on Japanese climbing fern. Control via prescribed burning is ineffective and may provide a fuel ladder to canopy trees due to L. japonicum's growth patterns (Minogue et al. 2009). Its underground rhizomes also allow for survival and fast regrowth of vegetation following a burn. Herbicide treatment has been shown to offer some promise, although concerns exist regarding damage to native vegetation (reviewed in Minogue et al. 2009; Bohn et al. 2011). Although biocontrol is another option, no such agents have been identified for this species, with all such efforts focused on *L. microphyllum* (Minogue et al. 2009). Due to the current lack of strong control methodology, it is possible that L. japonicum will continue to spread and displace native vegetation throughout the southeastern U.S. Importantly, a lack of information regarding L. japonicum genetic variation in the U.S. limits current control efforts. Knowledge of genotypic diversity, origin(s), and genotype distribution is important for controlling invasive plants, especially if there is preliminary evidence that control agents have differential success across multiple genotypes (Ward et al. 2008; Gaskin et al. 2011; Darling 2015; Sun et al. 2020).

In this study we aim to determine if there were multiple introductions of Japanese Climbing Fern, and if so, document the distribution of alternative haplotypes throughout the invaded range. We also aim to determine the source population(s) from the native range in Asia. These three aims are accomplished by sequencing chloroplast genomes from herbarium specimens throughout the distribution of L. japonicum in the U.S. and East Asia. Whole chloroplast genomes are easily obtainable from herbarium specimens of a wide age range (Alsos et al. 2020), and are analytically straightforward due to their uniparental inheritance (Gastony and Yatskievych 1992). Additionally, prior studies have shown chloroplast DNA sequence to be useful for identifying the number of introductions and characterizing the amount of genetic variation transported to the introduced range. Gaskin et al. (2005) found 41 different chloroplast haplotypes in Lepidium draba L., with 20 of these haplotypes found in the U.S. Hufbauer and Sforza (2007) examined Centaurea diffusa Lam. and Centaurea stoebe L. (Asteraceae), and found 11 haplotypes in Centaurea diffusa - 9 observed in the native range and only three in the introduced range. The C. stoebe sample set contained 11 haplotypes- 10 in the native range, and four in the introduced range (Hufbauer and Sforza 2007). Oduor et al. (2015) observed 32 haplotypes in Brassica nigra L., with 22 in the native range and 13 in the introduced range. Williams et al. (2005) observed 10 haplotypes in Schinus terebinthifolius G. Raddi- nine in the native range and two in the introduced range. The consistent observation of multiple invasive range haplotypes, albeit fewer than in the native range, suggest that multiple introductions are common but that they are not sufficient to transfer total native-range chloroplast genetic variation to the invasive range. These two observations therefore serve as null expectations for L. japonicum.

Methods

All samples were obtained from herbarium specimens via loans and in-person visits. Each *L. japonicum* herbarium specimen was examined to confirm species identification, and approximately one half of one leaflet was removed and stored in silica gel desiccant. Google Earth Pro (Google Inc. 2021) combined with online place name searches were used to georeference all specimens. DNA extraction was performed using the 96-well protocol presented in Beck et al. (2012). A Qubit model 2.0 fluorometer (Life Technologies, Eugene, Oregon) was used to establish sample DNA concentration with the double-stranded broad-range DNA kit. Samples were chosen for genomic library preparation based on geographic disparity and DNA concentration.

Library preparations were performed using the NEBNext Ultra II DNA Library Prep Kit for Illumina with the NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) (NEB, Ipswich, Massachusetts). Library preparation followed the protocol outlined in Saeidi et al. (2018). Samples with low library concentrations were re-amplified with universal Illumina primers (Saeidi et al. 2018). Following library preparation, 87 of the 98 samples were selected for sequencing. Unenriched libraries were sequenced with 150 bp paired end chemistry on an Illumina (Illumina, San Diego, California) NextSeq 550 at the University of Kansas Genome Sequencing Core. Trimmed (Trimmomatic- Bolger et al. 2014) chloroplast sequences were then aligned to a published 157,260 bp *Lygo-dium* chloroplast genome (Genbank accession number KC536645) (Gao et al. 2013) using Geneious (Biomatters, Aukland, New Zealand). Samples exhibiting less than 10,000 reads aligned to the *Lygodium* chloroplast genome were removed, as were four samples with highly divergent chloroplast genomes. These divergent

samples also displayed morphologies suggesting they represent other *Lygodium* species. Consensus sequences were formed using a threshold of 75% and nucleotides were called as ambiguous if coverage was less than five. Consensus sequences were aligned using MAFFT (Katoh et al. 2002). Following alignment, all nucleotide positions exhibiting ambiguities, gaps, and identical bases were masked. The average number of pairwise nucleotide differences between samples within both the native and introduced ranges was calculated in DnaSP version 6 (Rozas et al. 2017). A Templeton, Crandall, and Sing (TCS) network (Clement et al. 2002) was produced from the resulting masked alignment in PopArt (Population Analysis with Reticulate Trees) (Leigh and Bryant 2015). The relationship between specimen age and various downstream measures of success (library concentration, mapped reads, coverage) were evaluated with linear regression in R version 4.3.0 (R Core Team 2023).

Results

Of the 191 specimens selected for extraction, 189 yielded a measurable DNA concentration. DNA concentration ranged from 0 to 247 ng/µl (mean = 37.64 ng/ µl, \pm 41.08 ng/µl). The collection year of extracted specimens ranged from 1910– 2017 (mean = 35.05 years old, \pm 15.53 years old). DNA was successfully extracted from both the oldest specimen (137 years old), as well as the youngest specimen (3 years old). After selecting a subset of DNA extractions based on geographic disparity and DNA concentration, 87 samples were chosen for genomic library preparation and sequencing. Library concentration ranged from 1–22.6 ng/µl (mean = 6.08 ng/µl, \pm 3.92 ng/µl), and the relationship between library concentration and specimen age was not significant (R² = 0.00258; p = 0.640).

All sequencing reads are archived on the NCBI Sequence Read Archive (SRA) (BioProject ID #PRJNA1114707). The number of reads mapped to the reference ranged from 800–806,545 (mean = 174,398 \pm 166,618). Mean coverage ranged from 0.81–679.77 (mean = 129.74 \pm 134.85). Specimen age had a strong negative effect on both the number of mapped reads (R² = 0.189; p = 2.59e⁻⁰⁵) and mean coverage (R² = 0.218; p = 5.03e⁻⁰⁶). Even with this effect of specimen age, most samples yielded useable chloroplast assemblies, with 85 of 87 samples passing our threshold of >10,000 reads aligned to the *Lygodium* chloroplast genome. Consensus sequences of 74 *L. japonicum* samples with fewer than 10,000 ambiguities were aligned. These consensus sequences included 50 *L. japonicum* samples from the introduced U.S. range and 24 *L. japonicum* samples from the native Asian range (See Suppl. material 1).

Following removal of all ambiguities, gaps, and identical bases from the alignment, 35 SNPs remained. The TCS network (Fig. 1) exhibited 18 *L. japonicum* haplotypes. Sixteen haplotypes were found in the native range, with three present in the introduced range. A single haplotype dominated the invasion and was observed in 47/50 (94%) of U.S. specimens (present in all eight states sampled). It is also the only haplotype seen in South Carolina, Florida, Alabama, Georgia, Texas and Arkansas. Interestingly, this common haplotype was not observed in the native range. The second invasive haplotype was only found in Mississippi, with the third invasive haplotype present in Louisiana as well as China and the Philippines (Figs 1, 2). The average number of pairwise nucleotide differences between samples was higher in the native (4.812) vs. the introduced range (0.277).







Figure 2. Location of Lygodium japonicum invasive haplotypes. Colors match haplotypes shown in Fig. 1.

Discussion

We draw three basic conclusions from our results: (1) there were at least three introductions of *L. japonicum* into the U.S., (2) Japanese climbing fern exhibits a low diversity invasion, and (3) the native source population(s) of L. japonicum remain unknown. Our observation of three distinct invasive-range haplotype establishes that multiple introductions have occurred. The only other scenario that would lead to this diversity would be a single introduction comprising multiple haplotypes. We view this as less likely, given the possibility of a single-spore origin of the initial invasive population. Instead, the dominance of the common invasive haplotype suggests that it arrived first and spread throughout the U.S. before the arrival of the two remaining invasive haplotypes. This is supported by collection dates associated with specimens harboring these haplotypes. The common invasive haplotype was the first to be observed (1940, Florida), with the two less common haplotypes observed later (1965, Mississippi; 1996, Louisiana) (See Suppl. material 1). An early arrival of the common invasive haplotype is, of course, consistent with its geographic dominance, although the seemingly late arrival of the less common haplotypes could simply be due to the likelihood of observing a relatively rare sequence. Inferring multiple introductions is not unexpected, since these have been commonly observed in prior reviews of plant isozyme studies (Dlugosch and Parker 2008) and in a variety of investigations using chloroplast sequence data (McIvor et al. 2001; Gaskin et al. 2005; Williams et al. 2005; Hufbauer and Sforza 2008; Oduor et al. 2015; Holt et al. 2023). Multiple introductions did not, however, lead to high introduced-range genetic diversity. Only three haplotypes were observed in the U.S., one of which was found in 94% of U.S. samples. The dominance of this haplotype and the relatively low genetic distance between any of the three haplotypes found in the U.S. also led to a markedly lower average number of pairwise nucleotide differences within the introduced range relative to the native range. The only published study that has examined whole chloroplast genome diversity in a North American invasion is that of Salvinia molesta D. Mitch. (Holt et al. 2023), in which nine unique haplotypes were observed in the U.S. In that study, 21 U.S. samples were sequenced, so a unique haplotype was observed for every two samples sequenced. Three haplotypes were observed in 50 U.S. L. japonicum samples (1 for every 17 samples sequenced). Although this relative lack of diversity among the L. japonicum genomes we sequenced suggests that this is a low diversity invasion, comparisons to future studies of whole chloroplast genome diversity in U.S. invasions should be considered. Consistent with many other comparisons of native and introduced range chloroplast sequence data (McIvor et al. 2001; Gaskin et al. 2005; Williams et al. 2005; Hufbauer and Sforza 2008; Oduor et al. 2015) this level of diversity (3 haplotypes) was also a clear reduction from that seen in the native range (16 haplotypes).

Regarding native-range source areas, the only haplotype match was between one observed twice in Louisiana to one observed in China and the Philippines (Fig. 1). Although this suggests two possible source populations, the fact that we did not observe a match to the overwhelmingly common introduced haplotype suggests that the geographic source of the bulk of the invasion remains unknown. This lack of a match between the most common introduced-range chloroplast haplotype and a native-range haplotype is unusual, as such a match is frequently observed in similar studies (Saltonstall 2002; Gaskin et al. 2005; Williams et al. 2005; Hufbauer and Sforza 2008; Oduor et al. 2015). The lack of such a match could be due to insufficient native-range sampling, although it does suggest that the dominant introduced-range haplotype is relatively uncommon in the native range. An alternative explanation for this pattern involves a novel mutation, as the common invasive haplotype is only a single mutation removed from one observed in two Chinese samples (Fig. 1). We view this as unlikely, as such a mutation would have had to have occurred in the very narrow time window between initial introduction and the spread of this now novel haplotype throughout the invasive range.

Knowledge of genotypic diversity, source(s) and genotype distribution is important for controlling invasive plants, especially if there is preliminary evidence that control agents have differential success across multiple genotypes (Charudattan 2005; Gaskin et al. 2005; Morin et al. 2006; Ward et al. 2008; Gaskin et al. 2011; Darling 2015; Sun et al. 2020). Specifically, our finding of low genetic diversity overall and a single haplotype dominating the invasion suggest that a biocontrol solution for L. japonicum might be feasible. The strategy for finding a biocontrol agent usually involves searching within the native range of the invasive species for natural enemies that are both host specific and damaging to the invasive species (Roderick and Navajas 2003). In the case of the Japanese climbing fern, work on L. microphyllum serves as an example. Goolsby et al. (2004) identified several genotypes of lygodium gall mite (Floracarus perrepae Knihinicki & Boczek) from Australia and Asia. They concluded that mites had differing success on the invasive fern genotype; the mite genotypes that performed best came from regions where the native fern genotypes were most similar to the invasive genotype (Goolsby et al. 2004). In the case of *L. japonicum*, the fact of a low diversity invasion therefore potentially simplifies the process of identifying biocontrol genotypes. Ideally, knowing the source location(s) of the L. japonicum invasion would greatly narrow the search for appropriate natural enemy genotypes, and further work should expand native range sampling to discover the source region(s) for the common genotype observed in the U.S. invasion. Additionally, similar studies of invasive and native-range genetic diversity should be conducted with high-resolution nuclear data (Peterson et al. 2012). Until biocontrol methods are implemented, it is likely that *L. japonicum* will continue to spread and displace native vegetation throughout the southeastern U.S.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

All authors have contributed equally.

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

Sample information

Authors: Morgan L. Markley, Ethan Altergott, James B. Beck

Data type: xlsx

Explanation note: Information for the 74 *L. japonicum* samples used to construct the haplotype network.

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Research Article

Predicting the expansion of invasive species: how much data do we need?

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Abstract

Ecological niche models (ENMs) are a powerful tool to predict the spread of invasive alien species (IAS) and support the implementation of actions aiming to reduce the impact of biological invasions. While calibrating ENMs with distribution data from species' native ranges can underestimate the invasion potential due to possible niche shifts, using distribution data combining species' native and invasive ranges may overestimate the invasion potential due to a reduced fitness and environmental tolerance of species in invaded ranges. An alternative may be using the increasingly available distribution data of IAS as they spread their invaded ranges, to iteratively forecast invasions as they unfold. However, while this approach accounts for possible niche shifts, it may also underestimate the species' potential range, particularly at the early stages of the invasion when the most suitable conditions may not yet be represented in the distribution range data set. Here, we evaluate the capacity of ENMs to forecast the distribution of IAS based on distribution data on invaded ranges as these data become available. We further use dispersion models to assess the expansion process using the predicted potential distributions. Specifically, we used the common waxbill (Estrilda astrild) in the Iberian Peninsula as a model system. We built ENMs with 10×10 km grid cells distribution records cumulatively for each decade from 1960 to 2019, and yearly bioclimatic variables, to forecast the species potential range in the coming decades. Then, we assessed the performance of the models for each decade in forecasting the species' observed range expansion in the following decades and evaluated how the number of distribution records determined the quality of the forecasts. Finally, we performed dispersal estimates (based on species traits, topography, climate and land cover) to analyse the prediction capacity of models as their uncertainty may be reduced when projecting them to the next decades. Our results show that invasion-only ENMs successfully forecasted the species' range expansion over three decades after invasion, while dispersion models were not important in forecasting common waxbill expansion. Our study highlights the importance of constantly monitoring alien species, suggesting that iterative updating of ENMs with observed distribution data may accurately forecast the range expansion of alien species.



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Key words: Alien species, common waxbill, dispersal analyses, ecological niche models, *Estrilda astrild*, forecasts

Introduction

Biological invasions are among the most worrisome environmental problems in modern times (Díaz et al. 2019). The spread of invasive alien species (IAS) worldwide has been responsible for population declines of native species, changes in community composition (Bellard et al. 2016, 2021), alterations of ecosystem processes and functioning (Ehrenfeld 2010), disruptions of socio-economic activities (Diagne et al. 2021) and public health issues (e.g. Naeem et al. 2009; Fournier et al. 2019; Ogden et al. 2019). In a globalised world, the number of IAS is expected to increase (Seebens et al. 2021), as well as their potential impacts (Fournier et al. 2019; Essl et al. 2020), promoted by the increasing international wildlife trade and global changes (Scheffers et al. 2019; Naimi et al. 2022). As a response to this urgency, several international regulations and mechanisms have been implemented in recent decades to prevent the introduction and spread of IAS. This includes the establishment of a legal framework with specific legislation, such as the EU Regulation 1143/2014 on IAS (Regulation EU 2014). However, the successful implementation of these mechanisms requires the anticipation of new invasion areas which have been hampered by the lack of monitoring data on species distributions at adequate spatial and temporal resolutions (Santana et al. 2023). There is thus a need for a continuing effort to develop approaches, which may include ecological modelling tools, to accurately predict IAS expansion, in order to reduce both the ecological and socio-economic impacts of IAS.

Modelling and projecting the realised niche of IAS in the geographical space allows the identification of the areas at risk of invasion (Jiménez-Valverde et al. 2011; Guisan et al. 2014). The realised niche is part of the fundamental niche, i.e. the abiotic environmental space where a species can maintain a viable population and persist over time without immigration, which is then further limited by biotic interactions, dispersal capacity, or historical aspects (Soberón and Peterson 2005). This assessment is often done through correlative ecological niche modelling (ENM) (Peterson and Vieglais 2001; Jeschke and Strayer 2008; Jiménez-Valverde et al. 2008; Capinha and Anastácio 2011; Venette 2015; Sillero et al. 2021), which quantify species-environment relationships based on observed patterns of species distributions and environmental predictors (Franklin 2010; Peterson et al. 2011; Guisan et al. 2019; Sillero et al. 2021). A procedure of key practical importance concerns the geographical areas used to calibrate the ENMs. For IAS, these models can be calibrated using distribution data from the species' native range (Peterson et al. 2003), thus assuming that the species native distribution represents the entire suite of suitable environments (i.e., distributional equilibrium; Guisan and Zimmermann 2000; Araújo and Pearson 2005; Araújo et al. 2005), or at least, all suitable habitats where the species can disperse (i.e. pseudo-equilibrium; Anderson and Raza 2010; Sillero et al. 2021). However, species' realised niches may shift in new areas or periods (i.e. niche shift sensu Guisan et al. 2014), which implies that IAS will not be necessarily circumscribed to areas that are environmentally analogous to their native ranges (Peterson 2003; Jeschke and Strayer 2008; Elith and Leathwick 2009). This is because, when the environmental conditions change, or the species arrives in a new area, the drivers limiting the species' realised niche can change (e.g. the new area lacks a competing species or the species can now disperse to new habitats), enabling the exploration of new areas inside its fundamental niche (Sillero et al. 2022). Some IAS have shown marked climatic niche shifts during invasion (i.e., a divergence between climatic conditions in native and alien

ranges; *sensu* Broennimann et al. 2009), likely driven by adaptive changes enabling species to endure conditions that were previously unsuitable (Blossey and Notzold et al. 1995), i.e., a shift in its realised climatic niche (Sillero et al. 2022).

Considering the potential for realised niche shifts, previous studies have recommended calibrating ENMs using distribution data of IAS in both native and invasive ranges (Fitzpatrick et al. 2006; Broennimann 2007; Broennimann and Guisan 2007; Urban et al. 2007; Beaumont et al. 2009; Pili et al. 2020). While this approach potentially captures niche shifts as they emerge in invaded areas, the combination of native and invasive distribution data raises relevant practical and conceptual issues. The existence of higher-quality distribution data for the species in one range versus the other (Vanette et al. 2010), may require a reduction in spatial resolution, leading to information loss when merging both data frames (Jarnevich et al. 2022). Although spatial downscaling can be employed to enable modelling at a coarse resolution and projection at a higher resolution, this approach introduces uncertainty due to assumptions regarding the consistent relationships between coarse and fine-resolution data within the area, on the employed methods (Keil et al. 2013). On the other hand, and perhaps more importantly, the use of native distribution data may overestimate invasion ranges. This is because there are circumstances where invasive species may not be able to colonise similar environmental conditions to their native areas. Factors such as novel negative interspecific interactions (e.g., predators, parasites, competitors) (Sih et al. 2010; Dostal et al. 2013; Carthley and Banks 2018), genetic bottlenecks and founder effects, can drive a reduction in the species' environmental tolerances, and species dispersal capacity. These factors cannot be included directly in the native-based model, which will provide the maximum extent of the species distribution in the invasive range if the environmental conditions are the same (Jarnevich et al. 2022).

Invasion monitoring efforts are producing high-quality spatiotemporal data of spread for a large number of IAS in invaded ranges (e.g. Groom et al. 2019; Howard et al. 2022). Hence, given the challenge of reconstructing the invasive process over time for most species, an alternative is to use spatiotemporal invasion data to iteratively forecast invasions as they unfold. The issues raised by using native distribution data are overcome by restricting the calibration of ENMs to the region being invaded. However, any approach relying only on invasive distribution data for calibrating ENMs must acknowledge the likely underestimation of species' potential ranges, particularly at early stages of invasion, when most suitable conditions may not yet be represented in the distribution range data set. In this context, it is pivotal to clarify the data requirements to ensure accurate ENM for IAS, and particularly, the extent to which invasion-only distribution data can be used to accurately predict the expansion of IAS. Modelling the invasion over time will provide information about the routes used by the species during the expansion process.

Here, we evaluate the capacity of iterative calibration of ENM models based on invasion-only distribution data to predict the invasion potential and analyse the expansion process of IAS. We specifically explore the relationship between the number of species distribution records since establishment and the capacity of models to inform about the species' invasion potential. We also assessed the importance of accounting for the dispersal capacity of species to predict their expansion. To achieve these aims, we considered one of the most studied and successful avian invasive alien species, established in different environments and biogeographic regions worldwide: the afro-tropical common waxbill (*Estrilda astrild*). This species was first introduced to

Portugal in 1964, and has spread across much of the country and into part of Spain fed by further introductions (Reino and Silva 1998; Silva et al. 2002). Common waxbill is a small-sized (<12 cm) granivorous finch that forages in low grass vegetation, typically found in open agricultural fields near water bodies (Payne et al. 2020; Ribeiro et al. 2020). The invasive success of this gregarious, non-territorial species may be attributed to its breeding biology and interspecific relations (Ribeiro et al. 2020), including: i) a variable breeding season (Sanz-Aguilar et al. 2015; Payne 2020); ii) the ability to produce several broods a year (Burton and Burton 2002); iii) vagrant movements in search of suitable habitat (flight range<37 km) (Payne 2020); and iv) the lower prevalence of parasites in non-native ranges compared to native regions (Lopes et al. 2018).

Using a unique, high-quality, database on spatial dispersion of the common waxbill through the Iberian Peninsula over six decades (Reino and Silva 1998; Silva et al. 2002; Reino 2005; Sullivan et al. 2012), we applied a backcasting approach, fitting ENMs using distribution data available until the end of each decade and using the resulting model to project the distribution for the next decade. Then, we analysed how the number of observation records used in each ENM was related to the performance of the forecasts of species dispersal over time. Finally, because ENMs do not account for species' dispersal per se (Sillero et al. 2021; Sillero et al. 2022), we also implemented a species dispersal model over time considering a comprehensive set of species traits and climatic and landscape variables (Engler et al. 2012). We discuss these results in light of the amount of distribution data (i.e. length of the time series since establishment) needed for invasion-only ENMs and dispersal analyses to predict the invasion potential of IAS and iteratively forecast future invasions.

Materials and methods

Study area

The Iberian Peninsula (southwestern Europe), covers an area of 582,860 km² and mainly includes the continental territories of Spain and Portugal (Fig. 1). It is bordered to the southeast and east by the Mediterranean Sea and to the south, north and west by the Atlantic Ocean, and is separated from the rest of Europe by the Pyrenees in the northeast. The Peninsula has a high diversity of climatic conditions, influenced by both the Atlantic Ocean and the Mediterranean Sea, with a longitudinal gradient of precipitation and a latitudinal gradient of precipitation and temperature (Capel 1981). While the Mediterranean climate dominates most of the area, the Oceanic climate occurs primarily in major mountain ranges and isolated southern mountains (Sillero et al. 2009).

Common waxbill distribution data

We gathered historical data on the common waxbill expansion in the Iberian Peninsula since its first introduction in the 1960s. For this, we obtained presence data of the species in the continental territories from Sullivan et al. (2012), including the national and regional breeding bird atlases from Portugal and Spain, updated with all-year-round information from the eBird database (eBird 2019) and authors unpublished data (L. Reino). Presumed new human-mediated introductions were considered whenever an isolated presence of the species was identified or when introduction events were documented through publications or personal



Figure 1. Location of the study area in the Iberian Peninsula, southwest Europe (upper panel) and distribution of the common waxbill *Estrilda astrild* in each decade from 1960 to 2019 (bottom panel) and the accumulated number of presences for each decade (n = #, bottom right of each map).

communications. Following data compilation, we mapped all records onto a 10×10 UTM km grid of Portugal and Spain (Fig. 1). We then aggregated the data by decade, from 1960 to 2019. Therefore, we represented the species expansion over six decades: 1st - 1960–1969 (9 UTM grid cells), 2nd - 1970–1979 (57 cells), 3rd - 1980–1989 (163 cells), 4th - 1990–1999 (340 cells), 5th - 2000–2009 (760 cells) and 6th - 2010–2019 (1128 cells) (Fig. 1, Suppl. material 1).

Environmental data

We obtained yearly climate data for the temporal period covered by the distribution data from the EuMedClim Database (http://gentree.data.inra.fr/climate/; Fréjaville and Garzón 2018), which provides yearly climate data between 1901–2014 at 1 km resolution for Europe and the Mediterranean Basin. We considered the seven bioclimatic variables available from this source: bio1 - annual mean temperature; bio2 - mean diurnal temperature range; bio5 - Maximal temperature of the warmest month; bio6 - minimal temperature of the coldest month; bio12 - annual precipitation; bio13 - precipitation of the wettest month; bio14 - precipitation of the driest month). From these variables, to minimise cross-correlation between variables, we kept four variables that had an absolute value of Pearson correlation coefficient below 0.7 (Suppl. material 2: table S1, fig. S1): mean diurnal temperature range (bio2), minimal temperature of the coldest month (bio6), precipitation of the wettest month (bio13) and precipitation of the driest month (bio14). As our species occurrences were available at 10×10 UTM km grid squares, we aggregated the values of these variables to match this resolution.

Ecological niche models

We estimated the realised niche of the species (*sensu* Sillero 2011) every decade, considering, for each model, the species distribution data cumulatively since the first introduction in 1960 (Fig. 1, Suppl. material 2: fig. S2). For this, we assumed that there were no extinctions, which is a reasonable expectation given the 10×10 km resolution, allowing us to use the climate data from the corresponding decade. Then, we projected each model to the following decades (Suppl. material 2: fig. S2). For example, we used the model calibrated with data from the first decade (1960–1969) to project the species' potential distribution in each of the following decades, i.e., 1970–1979, 1980–1989, 1990–1999, 2000–2009 and 2010–2019.

We calculated realised niche models using Maxent v.3.4.4 (Phillips et al. 2006, 2017) following standard procedures (Sillero and Barbosa 2021; Sillero et al. 2021). All Maxent models used the same parameterization. Specifically, we used presence/background data representing the spectrum of environmental conditions available to the species as dependent variables (Phillips et al. 2009; Guillera-Arroita et al. 2014). Background data consisted of 9196 randomly generated points distributed throughout the entire study area, including pixels where the species occurred. Thus, background points are not equivalent to pseudo-absences (Phillips et al. 2009; Guillera-Arroita et al. 2014). Maxent output represents habitat suitability, ranging from 0.0 (not suitable) to 1.0 (suitable), in Cloglog format (Phillips, et al. 2017). Because Maxent includes stochasticity in the training data random selection, different model runs can lead to slightly different outcomes (Phillips et al. 2006, 2017). For this reason, we used the average of 10 distinct modelling events to obtain the final suitability

values for each decade, randomly selecting 70% of the occurrence records as training data and 30% as test data. The models used auto features, where different distribution functions are used depending on the sample size (Phillips et al. 2006, 2017).

We measured model discrimination performance using the area under the curve (AUC) of the receiver operating characteristics (ROC) plots (Liu et al. 2005) and True Skill Statistics (TSS; Allouche et al. 2006). The ROC plot was calculated by representing the sensitivity against 1-specificity for all possible thresholds. It measures the proportion of true positives against the proportion of false positives, i.e., the likelihood that the model will rank a randomly chosen presence higher than a randomly chosen absence. The AUC is an integral of the ROC curve. The AUC discriminates a species' model from a random model, with a value equal to or close to 0.5 corresponding to an accuracy similar to that of a random model and a value of 1 corresponding to a perfect discrimination accuracy. TSS is equal to sensitivity + specificity - 1 (Allouche et al. 2006). TSS ranges from - 1 to 1, with 0 corresponding to random classification. AUC and TSS increase with the extent of the study area and are correlated (Leroy et al. 2018). Additionally, we calculated a set of null models following the methodology by Raes and ter Steege (2007). For this, we generated 100 different datasets with the same number of random points as each dataset following a Poisson distribution. We calculated a Maxent model for each of these random datasets and obtained the AUC values of the ROC plots. Then, we compared the training AUC values of the species models with the ones calculated for the null models using the non-parametric Wilcoxon test. We calculated the null models in R 3.4.4 (R Core Team 2020) with the 'dismo' package (Hijmans et al. 2017). As AUC and TSS are correlated (Leroy et al. 2018), we calculated the null models using only the AUC.

We used the Boyce Index (Boyce et al. 2002; Hirzel et al. 2006) to measure the level of agreement between predictions generated by the model and actual observations (proportion of presences). The Boyce Index is specifically designed for presence-background algorithms and measures the extent to which model predictions differ from a random distribution of the observed presences across the prediction gradients (Hirzel et al. 2006). The index calculates the proportion between the number of presences predicted by the model that fall into a particular class and the number of presences expected under a random classifier, plotted against the corresponding suitability category. The habitat suitability range is divided into b classes (or bins) to calculate a predicted-to-expected (P/E) ratio (Fi), where P is the predicted frequency of evaluation points and E is the expected frequency of evaluation points. If the model is well calibrated, low suitability classes should contain fewer evaluation presences than expected by chance (Fi < 1) and high suitability classes should contain more evaluation presences than expected by chance (Fi > 1). A good model should have a monotonically increasing curve when P/E is plotted against the mean habitat suitability of each class. The index ranges between -1 and +1: positive values indicate a model where predicted presences are consistent with the distribution of presences in the evaluation dataset; values close to zero mean that the model is not statistically different from a random model; negative values indicate counter predictions, i.e., predicting lower suitability in areas where presences are more frequent. We calculated the Boyce index with ModEvA 3.10 R package (Barbosa et al. 2013).

We determined the contribution of each climatic variable in explaining the species' distribution using a jackknife resampling based on: (1) values of the training and test gain; and (2) of AUC values. The jackknife resampling comprises two steps: (1) the generation of a model with all climatic variables except one; and (2) the generation of univariate models, each using only one climatic variable. In each step, the jackknife analysis measures the change in training and test gain, and the AUC determines the importance of each variable. Using the results from each of these procedures, Maxent calculated an average percentage contribution of each climatic variable. We also calculated the permutation importance: for each environmental variable in turn, the values of that variable on training presence and background data are randomly permuted. The model is re-evaluated on the permuted data, and the resulting drop in training AUC is calculated, and normalised to percentages (Phillips et al. 2006). When variables interact, the variable contributions and the permutation importances are not equally ordered, preventing individual responses for each variable.

Ecological niche models validation over time

We further validated the ENMs for each decade and their respective projections by counting the number of presences classified as presences or as absences. For this, we categorised the continuous models into two categories by applying the threshold 'Maximum training sensitivity plus specificity' for the Cloglog output. We used the presence records of each decade and previous decades, i.e., cumulatively. The total number of presences used to validate the projections of each model was the same (9, 114, 160, 334, 752, 1120, see Suppl. material 2: table S4). We also validated the projections of all the models using the presences from the last decade (i.e. 2010's; 1120 presences, see in Suppl. material 2: table S5).

Dispersal analyses

Accounting for dispersal barriers/capacity has been pointed out as important to reduce uncertainty in future projections of species distribution (Engler et al. 2012). We estimated dispersal movements over time with the R package 'MigClim' (Engler et al. 2012), a cellular automaton model that simulates the dispersal of species in the landscape. MigClim uses ENMs as indicators of landscape permeability: the higher the habitat suitability index, the higher the permeability. We applied MigClim to each decadal Maxent model and respective projections (Suppl. material 2: fig. S2). Therefore, Migclim modelled how the species dispersed between Maxent models, i.e. from the first model to the next projections. The MigClim model considers short-distance and long-distance dispersal events, the type of dispersion through the landscape (using a continuous or a categorical Maxent model), propagule production probability, initial maturity age, and the presence of barriers. We considered three possible scenarios: i) no dispersal barriers; ii) weak barriers (i.e. barriers that can be transposed), and iii) strong barriers (i.e. barriers that cannot be transposed) (Table 1). We assessed the significance of dispersion models in forecasting the expansion of the common waxbill by visually inspecting the maps produced for each scenario.

Dispersal barriers were represented by elevation, land cover, and hydrological factors (Fig. 2). We obtained elevation data from the Shuttle Radar Topography Mission (https://www.earthdata.nasa.gov/sensors/srtm; Farr et al. 2007) at 90 m and aggregated it to 10×10 km cell resolution. Following the results obtained in previous studies in Iberia (e.g. Silva et al. 2002), we considered elevations higher

than 800 m as barriers to dispersion. We obtained land cover from the Global Land Cover 2000 dataset with 250 m of spatial resolution from the European Environmental Agency (https://www.eea.europa.eu/data-and-maps/figures/globalland-cover-2000-250m). We considered the land cover classes including tree cover as barriers to the dispersal, as E. astrild is mostly associated with open habitats (Payne et al. 2020; Ribeiro et al. 2020) (Suppl. material 2: table S2). To represent hydrological configuration, we considered the vectorial dataset of free-flowing rivers in Europe from the European Environmental Agency (https://www.eea.europa. eu/data-and-maps/figures/free-flowing-rivers-in-europe). River hierarchy ranged from 1 to 8, with a value of 8 corresponding to large watercourses such as main rivers and 1 to small, often intermittent streams. We calculated the average hierarchy of watercourses in each grid cell of 10×10 km. For this variable, we considered as barriers those grid cells with a hierarchy of 1, reflecting the species' association with permanent water courses (Ribeiro et al. 2020). The final layer of barriers corresponded to the combination ((elevation + land cover) x river hierarchy) of all barrier layers, resulting in a layer classified as 0 (without dispersal barriers) or 1 (with barriers) (Fig. 2). The parameters used in MigClim are shown in Table 1.



Figure 2. Variables (elevation, land cover, and river hierarchy) used to define the barriers to dispersal through the landscape, and the barriers used in MigClim to measure the dispersion across the landscape. Blue means barrier; white means no barrier.

Parameter	Value
Continuous mode	0
Number of environmental change steps to perform	6
Number of dispersal steps to perform within each environmental change step	10
Dispersal kernel: probability of colonising a directly adjacent cell	1
Long-distance dispersal frequency	0.0001
Minimum distance for long-distance dispersal in pixels	2
Maximum distance for long-distance dispersal in pixels	4
Initial maturity age of newly colonized cells	1
Propagule production probability as a function of cell age	1
Number of replicates	100
Barriers	No barriers / Weak / Strong

Table	1.	Parameters	and	values	used	in	dispersal	models.

Results

Common waxbill expansion patterns

The current geographic distribution of the common waxbill in the Iberian Peninsula spans most of the Iberian Atlantic coast, but also through large areas in Southern and Eastern Iberia extending to the Mediterranean coast, as far as Catalonia (Fig. 1). Although this species was initially introduced around the Lisbon region (Central Portugal) during the 60's, it rapidly spread in all directions during the following decade. Its geographic expansion was also enhanced by more recent and independent introductions, as in the Algarve (southern Portugal), during the 70's, and Andalucia (southern Spain) and later in other regions in eastern Spain, during the 80's (Fig. 1).

The expansion process was faster in the Central and Northern regions of Portugal (70's), whereas the spread in the south seemed to have been boosted by an additional introduction event in the Algarve in the same decade that enabled the colonisation of western Andalucia during the following decade (Fig. 1). This event appears to underlie the colonisation of almost all Iberian southwestern coasts, unifying the northern population - which had also started to colonise Alentejo (Portugal) from the Sado River valley in the 80's - with the southern population (Fig. 1). Spread eastwards was slower, and several areas remain uncolonized to the present, namely the mountainous regions of Northern and Central Spain (Fig. 1). Spanish populations have arisen from independent introductions and the expansion of Portuguese populations directly from the extensive area bordered by the Minho River in Galicia in the 80's, and through the Tagus and Guadiana River valleys in the Southern and Central regions by the 90's (Fig. 1).

Temporal changes in ecological niche projections

ENMs had test AUC values higher than 0.8 and significantly differed from random (Table 2, Suppl. material 2: table S3). All test TSS values were higher than 0.6 (Table 2). The Boyce index exceeded 0.85 for all decades, except the first (0.02). This indicates a consistent prediction of presences by the models, aligning closely with the distribution of presences in the evaluation dataset for all but the first decade, to which the model is not statistically different from random.

The variable with the highest contribution for the first decade (1960–1969), was the mean diurnal temperature range (bio2), whereas the minimum temperature of

the coldest month (bio6) was the variable with the highest contribution in the following decades (Table 3). The permutation importance of variables maintained the same order as the variable contributions (Table 3).

The areas identified as suitable widened over time, from the coastal areas towards the interior of the Iberian Peninsula (Fig. 3). Suitable areas based on data from the first decade (1960–1969) were enclosed within the vicinity of the introduction area around Lisbon (Portugal, Fig. 3). There was an abrupt change in the extent of suitable areas between the third and the fourth decades (1980–1989 and 1990–1999, Fig. 3). The increment in suitable areas stabilised in the fourth decade after introduction (1990–1999, Fig. 3).

Ecological Niche Models validation

Validation of the ENMs of each decade projected to the remaining periods (Fig. 4, Suppl. material 2: table S4) indicated the proportion of the presences correctly classified is >80% for the projections of all models, except for the projections for the last decade based on the models using distribution data of the first and second decades (44% and 50%, respectively). The same pattern was observed in the models using data from the past decade, so the results are presented only in the Suppl. material 2: fig. S3, table S5.

			0		
Decade	Training n	Test n	AUC	TSS	Boyce index
1960–1969	4	1	0.99 ± 0.010	0.83 ± 0.09	0.06
1960–1979	24	10	0.98 ± 0.010	0.91 ± 0.03	0.86
1960–1989	73	31	0.95 ± 0.010	0.80 ± 0.03	0.98
1960–1999	171	73	0.92 ± 0.010	0.71 ± 0.02	0.99
1960-2009	432	184	0.88 ± 0.010	0.63 ± 0.01	0.99
1960-2019	639	273	0.86 ± 0.010	0.60 ± 0.01	0.99

Table 2. Results of AUC, TSS, and Boyce index for models considering the accumulated presences since the first introduction.

Table 3. Contributions and permutation importance of the bioclimatic variables (mean diurnal temperature range (bio02), minimal temperature of the coldest month (bio06), precipitation of the wettest month (bio13) and precipitation of the driest month (bio14) of the Maxent models. Highest values of variable contribution and permutation importance for each model are highlighted in bold. Temperature, and particularly the minimal temperature, was the most important variable affecting the distribution of common waxbill for all models.

Decade	Variable contribution				Permutation importance			
	bio02	bio06	bio13	bio14	bio02	bio06	bio13	bio14
1960-1969	50.97	19.75	0.06	29.22	39.97	3.74	0.14	56.15
1960–1979	4.59	72.24	6.58	16.60	0.90	84.85	4.54	9.710
1960-1989	2.76	79.67	7.62	9.95	1.18	80.53	5.82	12.47
1960-1999	6.06	76.36	8.40	9.18	5.74	78.30	10.46	5.501
1960-2009	4.90	68.36	15.33	11.41	5.37	64.04	21.40	9.19
1960-2019	5.14	67.70	15.10	12.06	5.69	62.53	17.63	14.16

Dispersal analyses

The species' potential range accounting for dispersal capacity increased over time, driven by the results of ENM projections. In the first decade, the range deemed susceptible to colonisation was narrow, and almost all of the Iberian Peninsula was beyond reach. On the other hand, for the last decade, these areas were much wider (Fig. 5). Similarly



Figure 3. Results of cumulative ecological niche models projecting habitat suitability to the next decades. The suitability maps are organised as in Suppl. material 2: fig. S2: each decade is a row; models (light blue background) and projections (yellow background) are placed in columns. Habitat suitability ranges from dark blue (low suitability) to red (high suitability), following the turbo palette.

to what was verified for the ENM, the spatial patterns of the dispersal models have remained quite stable since the fourth decade (1990–1999). Visual inspection of the maps reveals that there were some differences when barriers were introduced in the dispersal models compared to when they were not (Fig. 5), but the results with weak or strong barriers are very similar. Dispersal models thus confirmed that the species was able to disperse over time following suitable areas identified by ENM.

Discussion

This study evaluates the use of increasingly available spatiotemporal data on IAS spread to iteratively forecast invasions as they unfold. The backbone of these forecasts were ENMs using detailed distribution data of the common waxbill expansion through the Iberian Peninsula over six decades. Our projections, based on invasion-range data, were



Figure 4. Percentage of presences incorrectly (red) and correctly (green) classified over time, for each model (blue background) and projection (yellow background). Validation for each model was conducted using the number of cumulative presences from the previous decade(s) (for details see Suppl. material 2: table S4). Our backcasting approach showed a high forecast capacity of EMNs after the 3rd decade following the common waxbill establishment (high % of correctly classified presences).

successful in forecasting the species' current distribution after three decades following its introduction. These results support the idea that ENMs can successfully forecast the species' range expansion, although they may have limited utility in the early stages of invasion, supporting the use of an iterative approach (Dietze et al. 2018), where models are recurrently updated with the species' most recent distribution data. This implies the need to constantly monitor IAS, which despite receiving long-standing support in invasion science and policy (Genovesi and Shine 2004; Büyüktahtakın and Haight 2018), depends strongly on the availability of resources (Groomet al. 2019).

Our results are in line with previous studies arguing that ENMs may underestimate the species' potential ranges (Liu et al. 2020), particularly at the early stages of the invasion when the most suitable conditions may not yet be represented in the distribution range dataset. The common waxbill is a tropical species sensitive to low temperatures (<15 °C) and wet weather conditions and inhabits open fields with tall grasses, often near water (Ribeiro et al. 2020). While detailed information on the distribution data in the native area is not available for analysis, existing data suggest that the distribution range of common waxbill in the invaded area generally falls within the climatic variation of its native African range, with



Figure 5. Results of dispersal models per decade and type of barriers (no barriers, weak barriers, and strong barriers). The colour sequence Blue -> Green -> Light Green -> Yellow indicates the dispersal of species over time in each decade. Yellow indicates areas where the species did not have time to arrive. Purple indicates areas where the species cannot occur because habitat suitability predicted by ENMs was low.

Occupied

Non

occupied

expansions observed into colder and rainier areas in Northern Iberia in recent decades (Ribeiro et al. 2020). Our projections based on invasion-only data failed to forecast the current species distribution using data from the first two decades after species introduction, likely because the species range was still not representative of the species' suitable environmental conditions (Araújo and Pearson 2005). The actual niche overlap of the species is probably contributing to the model accuracy of the last decades. However, the AUC has slightly decreased in the models for the last three decades probably due to the increment in the presences: as the species distribution range increases, the species has a more generalist character, making it harder to predict (Guisan and Thuiller 2005; Sillero et al. 2021).

Models disregarding the species' global distribution provide worse results than full distribution models (Barbet-Massin et al. 2010; Capinha et al. 2011; Jarnevich et al. 2022). This is because ENM algorithms assume that the species distribution data used is a good representation of the species' environmental requirements (Sillero et al. 2021). In other words, the algorithm assumes that the data used represents a species in equilibrium with the environment, i.e. the species occupies all available suitable habitats where it can disperse (Guisan and Thuiller 2005; Anderson and Raza 2010; Sillero et al. 2021). Distributional data representing only a portion of a species' global range may fail to capture all the suitable conditions where the species can thrive, potentially leading to an underestimation of its potential range. Therefore, modelling the realized niche of an expanding alien species presents significant challenges (Ficetola et al. 2005). It is expected that the ENM for a particular period will fail to forecast imminent range expansion stages, although this does not mean that the ENM is inaccurate (Barbet-Massin et al. 2018). The ENM for that particular period can be accurate, but the ENM does not have enough information to predict the upcoming dispersion process. This was our case: the increment of new suitable areas in ENM projections stabilised from the third decade after introduction (1990-1999), i.e., only after three decades of dispersion, the species' occurrence data were representative of the species' environmental requirements. The abrupt change between the third and fourth decades might be strongly influenced by new introduction events in the third decade that occurred in regions environmentally different from previous ones, particularly in the East of the Iberian Peninsula. These results thus suggest that modelling expansion based on the early stages of introduction may provide limited results, demanding the interactive recalibration of models as new distribution data becomes available.

Contrary to expectations, our results suggest that barriers to dispersion were not insuperable by the common waxbill, although they might be important for other species with lower dispersal capacity. MigClim considers long dispersal events where the species reaches new locations without human intervention. In that case, the species only needs to arrive at a pixel with enough suitability. The few differences found in the projections using the ENM-only and dispersion models (with strong and weak barriers) indicate that the species was able to disperse over time following the suitable areas predicted by ENMs. In line with the commonly observed lag period during the invasion process (generally attributed to the exponential growth process, stochastic extinction of propagules, or an evolutionary modification of species following establishment, Sakai et al. 2001), previous studies have suggested that the dispersion capacity of the common waxbill across Iberia was very low in the first decades after the first introduction (e.g., Silva et al. 2002; Reino 2005). Justifications for this lag period for the common waxbill relied on the stochastic extinction of propagules due to the absence of favourable habitat conditions (i.e. agricultural fields near water bodies, Ribeiro et al. 2020) out of the areas where the species was first introduced. This was based on the slower colonisation process in the southern regions of Portugal, where initial populations were very small and limited to the Tagus valley around Lisbon and the westernmost region of Iberia (Portugal) and an acceleration after the 80s. According to these studies, the additional introductions across Iberia might have jointly fostered higher dispersion rates to new areas, suggesting that the dispersal capacity of the common waxbill in Iberia is a combination of both habitat suitability and propagule pressure. However, our results suggest that this is likely to be a consequence of insufficient data to capture the species' environmental requirements during the first decades, as they projected a potential for expansion lower than the real one.

While the common waxbill has been a highly successful avian invader across various continents and islands, its spatial spread appears more limited in comparison to other, predominantly older, Palearctic invaders worldwide, such as the European starling (Sturnus vulgaris) or the house sparrow (Passer domesticus). However, it seems to show a rather eclectic adaptation as these last two species colonise a great diversity of open and semi-open habitats, but not limited to human-made habitats (e.g., agricultural habitats, gardens), but also to wetlands. Probably, its expansion is more comparable with a more recent invader: red-billed leiothrix (Leiothrix lutea) in Europe. Though this species is more associated with forest habitats and is likely to be more limited to tree-based habitats, it has been a very successful and established invader with populations in several European countries and regions (Pereira et al. 2019). It is worth noting that both the European starling and the house sparrow have much older introductions, with established populations in regions like North America and Australia. For instance, the European starling was successfully introduced to multiple areas during the same period, as in Australia (Stuart et al. 2023). Variances in propagule pressures may offer a partial explanation for differences in range expansion.

Conclusions

Accurately anticipating the expansion of IAS is key to ensuring the successful implementation of preventive and mitigation actions. Forecasting invasions by means of different quantitative methods and modelling strategies have been used in the last decades, and new approaches are constantly emerging (Peterson 2003; Reino et al. 2009; Jiménez-Valverde et al. 2011). However, predictions may be severely compromised by different methodological options and their specific limitations. ENMs are powerful tools for predicting the spread of IAS and guide management. Although ENM enables predicting and evaluating biological invasions, it is often compromised by the amount (time-series length), quality (spatial and temporal resolution) and availability (data access) of distribution data in both native and invaded ranges. Our study evaluates the capacity of ENMs based on spatiotemporal data of invaded ranges only to forecast the potential distribution of IAS using common waybill dispersion analysis as a case study. We demonstrate that invaded range-only data may be used to accurately project the expansion of alien species in novel regions if enough time (at least three decades in our study model) is given to allow the species to expand and occupy the most suitable conditions. These results indicate that the invaded ranges-only ENMs are of limited utility in the early stages of invasion, while they clarify the need

for using an iterative approach where models are recurrently updated with the most recent distribution data of the species since establishment. This approach will contribute to a better understanding of climatic niche changes during the expansion process of alien species, and offers a solution to managers and scientists dealing with the scarcity and asymmetry of distribution data available for alien species worldwide, in their native and invaded ranges. Our study helps solve a much-discussed conundrum and offers a practical solution to better guide management actions and significantly improve stakeholders' ability to halt biological invasions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Data availability

EuMedClim Database: http://gentree.data.inra.fr/climate/. Shuttle Radar Topography Mission: https://www.earthdata.nasa.gov/sensors/srtm. Global Land Cover 2000 dataset: https://www.eea.europa.eu/data-and-maps/figures/global-land-cover-2000-250m. Free-flowing rivers in Europe: https://www.eea.europa.eu/data-and-maps/figures/free-flowing-rivers-in-europe. Estrilda astrild oc-currence data: Suppl. material 1. R Code used to run the analyses: Suppl. material 3.

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Supplementary material 1

Oldest records of common waxbill *Estrilda astrild* for each 10×10 UTM km grid cells of Portugal and Spain

Authors: Joana Santana, Neftalí Sillero, Joana Ribeiro, César Capinha, Ricardo Jorge Lopes, Luís Reino Data type: csv

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Link: https://doi.org/10.3897/neobiota.95.122335.suppl1

Supplementary material 2

Supplementary figures and tables

Authors: Joana Santana, Neftalí Sillero, Joana Ribeiro, César Capinha, Ricardo Jorge Lopes, Luís Reino Data type: docx

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Supplementary material 3

R code scripts used to run the analyses

Authors: Joana Santana, Neftalí Sillero, Joana Ribeiro, César Capinha, Ricardo Jorge Lopes, Luís Reino Data type: zip

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Research Article

Assessing ongoing risks and managing detections of non-native invertebrates in the Antarctic Region

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Abstract

The continent of Antarctica has remained relatively free of the impacts of invasive species to date. However, Antarctica is under increasing anthropogenic pressure from human activity and climate change, elevating the risk of alien species introductions. Scientific research and the maintenance of research stations by Antarctic Treaty Parties requires the transfer of large amounts of equipment and cargo, which can harbour biosecurity risk material. Here, we assess two decades of data collected by the Australian Antarctic Division on the detection of biosecurity risk material in its facilities and vessels, both during transport and in Antarctica. We use these data to identify emerging risk species or pathways, to compare the variability in detections over time and to construct a consequence table to facilitate effective responses and resource allocation to future detections, translating our research findings into guidance for decision-makers. We find that, despite the development of policy instruments, monitoring and management for the prevention of alien species introductions to Antarctica, the risk of introductions is ongoing. We highlight areas of concern, including the transport of live spiders and the continuing potential for cargo to harbour biosecurity risk material and the benefit of ongoing training and investment and support for staff and expeditioners in the reporting of non-native species detections. Finally, we provide tools and recommendations for decision-makers and on-ground managers in the Antarctic biosecurity space, based on our research. Future studies on the establishment risk of commonly transported species would assist in improving these tools.

Key words: alien species, Antarctica, biosecurity, non-native species management

Introduction

Antarctica is the last continent on the planet that remains relatively free of the impacts of invasive alien species (Leihy et al. 2023). However, the Antarctic is experiencing increasing anthropogenic pressure from both human activity and climate change and new and uncertain threats are emerging, such as the transformation of habitats as a result of expanding ice-free areas (Lee et al. 2022a). Annex II to the Protocol on Environmental Protection to the Antarctic Treaty (Article 4) states that no non-native species shall be introduced to the land, ice shelves or water of



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Copyright: © Isabelle R. Onley et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0) Antarctica without a permit (Antarctic Treaty 1991) (noting that this wording was intended to prevent intentional introductions and there is some uncertainty in terms of liability for accidental introductions (Hughes and Convey 2014)). Nevertheless, the unintentional transport of species and biological matter that are not native to the Antarctic and sub-Antarctic area, or biosecurity risk material (BRM) (which may include plant, animal and insect material, as well as pathogens, soil and water), continues to occur.

Non-native species are detected at various stages along transportation pathways to Antarctica, including within the cargo and packing facilities of national operators, on ships or planes en-route to Antarctica and within the station buildings and facilities on the sub-Antarctic islands and the Antarctic continent (Hughes et al. 2010, 2011; Houghton et al. 2016, 2019; Bergstrom et al. 2018; Remedios-de León et al. 2021). Antarctica's terrestrial communities have low species richness, simple community structure and narrow habitat ranges and the growing pressures of a changing climate makes them vulnerable to invasive species impacts (Convey et al. 2006). Biological invasions of Antarctica have resulted in the establishment of a number of non-native species in the warmer regions, in particular the Antarctic Peninsula and its islands, and the risk of the introduction of BRM to Antarctica continues to grow under increased anthropogenic pressure and climatic shifts (Hughes et al. 2015; Bergstrom 2022). The application of strict biosecurity measures, coupled with surveillance for high risk species by all stakeholders across Antarctica, is of paramount importance to reduce BRM introductions and invasions on the continent (Hughes et al. 2020).

To address the risk of non-native invasive species becoming established in Antarctica, a number of policies and guidelines have been developed for Treaty Parties. A primary resource for all Parties is the Committee for Environmental Protection (CEP) Non-Native Species Manual, which outlines key guiding principles to prevent unintended introductions of non-native species to the Antarctic Region, and the movement of species between biogeographic zones within Antarctica (Committee for Environmental Protection 2019b). Another key resource is the CEP Five-year Work Plan, released in 2019, which highlights the introduction of non-native species as a major priority from amongst a suite of environmental issues and concerns (Committee for Environmental Protection 2019a). Further, the Council of Managers of National Antarctic Programs (COMNAP), in conjunction with the Scientific Committee on Antarctic Research (SCAR), released a checklist for supply chain managers to assist in reducing the risk of transfer of non-native species to Antarctica by the National Antarctic Programs (Council of Managers of Antarctic National Programs 2010). Many of the Treaty Parties have also conducted research and developed individual biosecurity policies and protocols (e.g. British Antarctic Survey Biosecurity Regulations (British Antarctic Survey 2023)).

Australia has a long-standing history in Antarctica. Currently, Australia operates three active research stations on the continent and multiple planes and ship voyages to the continent throughout the summer season. Australian researchers have invested considerable effort in monitoring BRM in Australian facilities in the 21st century, particularly through the Aliens in Antarctica programme, an International Polar Year Program supported by multiple national operators, including the Australian Antarctic Division (AAD) through the delivery of the Australian Antarctic Program (AAP) (Whinam et al. 2005; Convey et al. 2006; Lewis et al. 2006; Chown et al. 2012; Huiskes et al. 2014; Houghton et al. 2016). In 2004, the AAD established an internal database to record environmental incidents, including BRM and, in 2013, they set up a new cargo handling and biosecurity facility to more effectively combat the inadvertent transport of non-native species (Houghton et al. 2016). In 2019, an environmental code for participants in the AAP was published which highlights personal responsibility for biosecurity, and the AAD's current cargo biosecurity standard operating procedures were authorised.

The Aliens in Antarctica programme identified food as a high-risk vector for non-native species introductions to the Antarctic Region and cautioned that the transport of BRM would likely continue without the improvement of ship-based management, constant vigilance and surveillance across the transport pathway. Nearly a decade has passed since monitoring under the Aliens in Antarctica programme ceased. Currently, biosecurity at Australian Antarctic cargo facilities consists of deep cleaning, sight inspections before and after packing of cargo, rodent baiting and trapping in the facilities, as well as internal and external fumigation of packed cargo containers with a non-residual insecticide. All of these steps are preceded by an ongoing supplier awareness and education campaign on the importance of biosecurity. During transport and on arrival at Antarctic stations, biosecurity visual inspections are made at the time of unpacking cargo at the destination, in addition to routine searching and trapping along the transport pathway and passive baiting and trapping at AAD facilities in Antarctica. Sub-Antarctic Macquarie Island is home to Australia's fourth research station and has additional biosecurity requirements due to the AAD's responsibility to the department of Natural Resources and Environment Tasmania (NRE) to manage the environmental impact of its activities. Furthermore, the sub-Antarctic environment has an increased risk of non-native species establishment due to its warmer, wetter climate and increased rate of human occupancy relative to the continent (Frenot et al. 2005; Leihy et al. 2023). Particular attention is paid to break bulk cargo (i.e. individual cargo not stored in shipping containers) and cargo containing potential refuge for invertebrates, as well as the risk of rodent re-introduction following the success of rodent eradication in 2011 (Springer 2016). Consequently, on voyages bound for sub-Antarctic Macquarie Island, biosecurity detection dogs are used to search the cargo packing facility and inspect personal effects for rodents.

BRM detections in AAD facilities or vessels are primarily reported using the AAD's electronic environmental incident reporting system. If BRM is found, expeditioners, crew and staff are required to contain, treat and remove the hazard. They are then required to report the incident and monitor the area for any ongoing or additional BRM. Where required, follow-up actions and specialist advice from the AAD are recorded in the incident report. Although all incident reports receive a risk rating once reviewed by the AAD, to date, there has been no standard rapid response risk assessment protocol to assess each BRM detection on-ground at Antarctic stations and guide a consistent, appropriate response. As such, BRM detections during AAP activities in the Antarctic Region (particularly at Macquarie Island) are often treated as high-risk incidents. This can result in the halting of offloading or unpacking procedures for long periods of time. There is a clear need for a framework to define what is high and low risk BRM as they are detected on-ground in the AAD's operations.

Given that the amount of human and cargo traffic to Antarctica continues to increase, there is an increasing risk of non-native species being transported to the Antarctic Region (Hughes et al. 2020). This study aims to identify emerging risk

species or pathways that exhibit high propagule pressure. Further, we compare the variability in BRM detections over time with the uptake of policies and protocols designed to manage the risk of BRM to gauge their effectiveness. Finally, we use records of BRM detections from the last two decades to construct a consequence table to facilitate more effective responses and resource allocation to detections during the transport and offloading of cargo at AAD stations, translating our research findings into guidance for decision-makers.

Methods

Since 2004, scientists, staff and expeditioners on AAD voyages and stations have recorded non-native invertebrates and other BRM, such as plant matter or soil, through the environmental incident reporting system. All reports made relating to BRM detections since 2004 were extracted from this database. These reports were in relation to the four Australian Antarctic stations (Casey, Mawson, Davis and Macquarie Island), as well as vessels and aircraft travelling to these stations, noting that some vessels were chartered from other national operators. The notes of each report were read to determine (where possible) the location and habitat (e.g. consumables, cargo) of each BRM detection and whether the BRM comprised living or dead material. The month and year of detection was also extracted. These data were manually filtered to eliminate any duplicate reports or reports that did not relate directly to BRM. Reports made in the year 2023 were removed, to ensure that only complete years were captured through the study period. We present data on all forms of BRM reported; however, given that the majority of the reports pertained to invertebrates, only invertebrates were considered in the downstream analysis.

Sensu Houghton et al. (2016), where reports in the database identified the BRM as a "spider", "fly" or "moth" or other distinctive forms, the report was categorised by the appropriate taxonomic order (e.g. Araneae, Diptera or Lepidoptera). If no identification was provided in the report or if the identification was not deemed reliable due to the specimen having a non-distinct form, identification to taxonomic order level was made by studying supplementary photographs (where available). Identifications were made by MJH. If no photograph or formal identification was available and the specimen was non-distinct, the report was classified as "unreported". Where possible, each detection was classified as "live" or "dead", based on the information presented in individual reports. Pupae, egg sacs and cocoons were considered "live" only if found alongside live adult invertebrates. Live detections were then used to identify trends, as dead invertebrates were assumed to have died during the fumigation of cargo, in which case they were successfully managed by biosecurity protocol.

To identify non-linear trends in the BRM data over time, we implemented a generalised additive mixed model (GAMM) using the R package "mgcv" v.1.8-42 (Wood 2017). The number of detections per year was modelled over time, with year as the smoothing parameter and a Poisson response as appropriate for count data. Analyses of temporal patterns of BRM detections must take into account the intensity of human activity, as this is likely to increase the risk of non-native species introduction (Hughes et al. 2020). Therefore, as a proxy to account for the degree of human traffic and amount of cargo entering AAD facilities each year, the number of voyages per year were sourced from the AAP website (https://antapps.aad.gov.au/public/schedules/). Voyages were included if they stopped at one or more AAD station and were assigned to the year that they departed their home port.

The number of voyages per year (ranging from three to sixteen) was then used as an offset in the GAMM model.

To construct a consequence table that can be used to respond appropriately to invertebrate BRM detections during transport and offloading procedures, we designed a consequence matrix (modified from Fletcher (2015)), based on common BRM detections reported in the AAD environmental incident database and factors influencing the probability of a biological impact of BRM in Antarctica. Using a manual inspection of the reports within the database, a consequence table was designed with four levels of impact – minor, moderate, major and severe – which were assigned a score from 1 to 4. The criteria for the consequence of a biosecurity failing were informed by past reports, logical assessments and literature. We present the consequence table designed for continental Antarctica – sub-Antarctic Macquarie Island's environment requires an alternative approach due to the more habitable environment and higher risk of establishment of non-native species.

Results

Between 2004 and 2022, 251 BRM detections were reported in the environmental incident database, including amphibians, birds, mammals, plant matter and soil (Table 1). The dataset contained 205 reports of invertebrates that were used in subsequent analyses, 42 (20%) of which were previously included in Houghton et al. (2016). The four most prevalent invertebrate orders reported were Araneae, Coleoptera, Diptera and Lepidoptera, with Araneae and Diptera emerging as the most common taxonomic orders. Of these four taxonomic orders, Diptera and Lepidoptera were most commonly found in consumable supplies (such as fresh vegetables), while Coleoptera and Araneae were primarily reported as being found in cargo (Fig. 1A). Approximately 86% of reports documented detections at AAD stations, while the remainder occurred on ships travelling to the stations (with the exception of one pre-departure report which detailed contamination of fresh food at Tasmanian Shipping Supplies) (Fig. 1B) (for a full summary of locations, see Suppl. material 1). The highest number of reported BRM detections was at Casey Station (84 detections) (Suppl. material 1). Specimens from the four most prevalent taxonomic groups were more commonly reported alive than dead and were most often reported in the adult life stage (Table 2) (Fig. 1C).

Type of biosecurity risk material	Count	Percentage
Invertebrate	205	81.67%
Plant matter	25	9.96%
Feather	6	2.39%
Mammal	5	1.99%
Invertebrate sign (web/nest)	4	1.59%
Bird nest	3	1.2%
Amphibian	1	0.4%
Bird	1	0.4%
Soil	1	0.4%
Total	251	100%

Table 1. Summary of reports of biosecurity risk material made by AAD crew and expeditionersbetween 2004–2022.



Figure 1. Proportion of biosecurity risk material (BRM) detections reported in various habitats (**A**), locations (**B**) and detection mortality status (**C**).

Table 2. Percentages of four most prevalent invertebrate groups reported alive, dead or unreported, along with life stages (pupae, cocoons and egg sacs were considered live if found in the presence of live adults).

Group	Alive	Dead	Unreported	
Araneae	43.90%	36.59%	19.51%	
	(94% adult, 6% egg sacs)	(94% adult, 6% egg sacs)	(72% adult, 14% egg sacs, 14% unreported)	
Diptera	50%	30.95%	19.05%	
	(95% adult, 5% unreported)	(92% adult, 8% unreported)	(87.5% adult, 12.5% larvae)	
Coleoptera	47.6%	33.4%	19%	
	(100% adult)	(100% adult)	(50% adult, 50% unreported)	
Lepidoptera	46%	40.5%	13.5%	
	(82% adult, 18% eggs/pupa/cocoon)	(93% adult, 7% larvae)	(80% adult, 20% pupa)	

Live detections over time (Fig. 2A) were analysed using a GAMM, with annual number of voyages as an offset (Fig. 2B). The model indicated a significant variation in BRM detections over time (p < 0.001, df = 8.7); however, it had an adjusted R² value of -3.81, indicating that the model fitted the data poorly and is less accurate than the mean value of the dataset over time (i.e. a straight line) (Ozili 2023). Examination of the residuals indicated a high degree of variance from the model. An increase in reported live detections can be observed between 2010 and 2015, with the number of reports stabilising in recent years.

Consequence table

A consequence table to categorise BRM detections from minor to severe, based on their potential for impact, was developed. Examples of commonly reported BRM detection events are provided for each level of consequence, both during transport and during offloading procedures on arrival (Table 3).



Figure 2. Number of live detections reported in the AAD environmental incident database over time (**A**) and GAMM analysis of detections with number of voyages per year as an offset (**B**) (95% CI).

	Level of Consequence						
	Minor - 1	Moderate - 2	Major - 3	Severe - 4			
Definition	Biosecurity risk material reaches Antarctica, but cannot survive in the environment.	Biosecurity risk material survives for a short time in the environment.	Biosecurity risk material survives for an extended period of time in the environment.	Biosecurity risk material survives and reproduces in the environment.			
Event –Transport Pathway	• Dead invertebrates found in areas known to be fumigated/treated	• Dead or living invertebrates found in unfogged areas/cargo/food.	• Small number (< 10) of live crawling invertebrates found on cargo/plant equipment or on vessel	• Infestation (> 10) of live invertebrates found on cargo/ plant equipment or on vessel			
		• Includes spider webs/egg masses/insect larvae	• Live winged invertebrates found on cargo/plant equipment or on vessel				
Event – Offloading Procedures	• Dead invertebrates found in areas known to be fumigated/treated	• Dead or living invertebrates (less than 10) found in unfogged areas/ cargo/food	• Egg masses/insect larvae found	• Infestation of live invertebrates within station (e.g. fresh food, dry goods, sewerage) – repeat event			
		• Includes spider webs	• More than 10 live or crawling invertebrates found within station (e.g. fresh food, dry goods, sticky traps) – single event only	• Live invertebrates found external to station			
Example Report	• Casey, 23/01/2021 – Alien invertebrate (moth)	• Mawson, 26/07/2018 – Spider found in science container	• Casey Station, 27/12/20 –Unknown alien larvae in celery	• Davis, 27/05/2014 – Live critters in hydroponics			
	• Description: "Around 1630 hrs on Thursday 21-Jan- 2021, an alien invertebrate (dead moth) was discovered in a shipping container between	• Description: "Whilst during fit-out in new science containers, a dead spider was found hanging in its web. Others were searched for but	• Description: "Around 1600 hrs on Friday 25-Dec- 2020 in the Casey Mess, a small number of unknown alien larvae were detected	• Description: "Small dark critters found in hydroponics. They appear to live on the surface of the water and on the side of the tubs".			
	drums of A1K during the annual resupply operation".	no others located".	in celery stems that were being chopped".	• Note: Supplementary photo indicated that the invertebrates were springtails.			

Table 3. Consequence table for BRM detections along the transport pathway and during offloading procedures at continental Antarctic stations.

Discussion

We have observed an increase in the number of live detections of BRM along the Australian invasion pathway to Antarctica between 2010 and 2015, despite the advent of the CEP Non-Native Species Manual in 2011. A similar trend is apparent in detection data from Scott Base, an Antarctic base operated by New Zealand (Newman et al. 2018). Live invertebrates have continued to be detected at Australian Antarctic stations, indicating they have survived biosecurity treatment and the environment during the journey. Although the AAD established a new cargo facility and implemented a comprehensive biosecurity plan in the early 2010s, no consistent decrease in BRM detection is apparent in the data - though detections appear to have remained relatively consistent in recent years. It is notable that the CEP's 5-year Work Plan was released in 2019 and prioritised preventing the introduction of non-native species to Antarctica. Other relevant policy and protocol introduced by the AAD and AAP around this time include the environmental code for participants in the AAP (2019) and the current cargo biosecurity standard operating procedures (2019), which have contributed to enhanced biosecurity measures at the AAD's cargo facility in recent years. These policy instruments likely resulted in increased awareness amongst staff and expeditioners of the importance of catching and reporting non-native species.

It is difficult to draw robust conclusions from the GAMM analysis and identify direct causes for trends in the time series given the negative adjusted R² value and poor model fit, likely a result of the short time span (< 20 years) and relatively low sample size of this dataset. Further, a number of other factors may have influenced the number of BRM detections beyond policy and protocol. For example, no increase in reported live detections since approximately 2015 could be indicative of a change in search effort or reduced incentive to report BRM in the online system, such as reduced capacity amongst logistics staff and expeditioners to actively search for BRM during that year. Furthermore, we have no measure of surveillance effort for this period. While concern and vigilance for non-native species along the invasion pathway to Antarctica by National Antarctic Programs was heightened in the 2010s (Hughes and Pertierra 2016), priorities may have shifted with the operational and logistics challenges of the COVID-19 pandemic (Hughes and Convey 2020; Liggett et al. 2023). Notably, in a number of incident reports, the habitat of entrainment, mortality status and taxonomic form are not provided, limiting the potential for the identification of recurring risk pathways. Users of the environmental incident reporting system may, therefore, benefit from regular training, the introduction of mandatory fields to the incident reporting forms (including photographic evidence) and/or the supporting specimen identification materials, of a generalist nature, embedded within the reporting protocols.

Analysis of reports on BRM detections made using the AAD environmental incident database indicate that the most prevalent taxonomic orders are Diptera (flies) and Araneae (spiders). The invasive potential of flies in Antarctica has received ongoing attention in the scientific literature (Hughes et al. 2005; Volonterio et al. 2013; Potocka and Krzemińska 2018; Pertierra et al. 2021; Remedios-de León et al. 2021), but to date, there has been little assessment of the invasive potential of arachnids in the Antarctic Region (but see Pugh (2004)), noting that the Antarctic continent has no native spider species. The most common habitat of entrainment reported for Araneae is cargo and a high proportion of individuals

are found alive despite insecticide fogging of cargo within AAD facilities (Bergstrom et al. 2018). Houghton et al. (2016) identified live spiders reaching research stations as a notable result of their study - however, the rate of detection of live spiders in the AAD database remains at ca. 0.9 live spiders per year after 2013 when Houghton et al.'s study concluded, a slight decrease from ca. 1 per year prior to 2013. Biosecurity measures may, therefore, still require improvement if the Antarctic Treaty is to be adhered to and the risk of biological impact by spiders as a result of human activity in Antarctica is to be further reduced. Spiders introduced to Europe in containers or packaging material have been found to have a high establishment rate and it is generally believed within the pest control industry that fumigation is not always effective for spiders (Vetter et al. 2014; Nentwig 2015). A study on brown recluse spiders (Loxosceles reclusa) in the United States of America required fumigation of sulphuryl fluoride at ten times the required fumigation rate for drywood termites to effectively control spider infestations, with a higher dosage likely necessary to control egg sacs (Vetter et al. 2014). The high prevalence of non-native spiders found on cargo and alive, in or en-route to Antarctica indicates that further control measures may be required, such as more intense fumigation and the use of oil-based aerosols or dry-heat treatment to target egg sacs (Vetter et al. 2016; Hayasaka et al. 2021) – although it must be acknowledged that the risk of establishment of spiders in the Antarctic environment may not be significant.

Uncertainty remains as to which non-native invertebrates will survive in Antarctica; non-native springtails and mites are known to survive in the soils of the Antarctic Peninsula (Greenslade et al. 2012; Russell et al. 2013; Hughes et al. 2015), while others, such as the black fungus midge Lycoriella ingenue at Casey Station (Hughes et al. 2005), persist synanthropically within station buildings, where conditions are more mild than the external environment. The fate of these synanthropic species is uncertain, but at least one non-native fly on the Antarctic Peninsula (associated with research stations) is expected to have established in the natural environment and previously synanthropic species have become naturalised on several sub-Antarctic islands, including Macquarie Island (Frenot et al. 2005; Phillips et al. 2017; Remedios-de León et al. 2021). Whether spiders transported to Antarctica have the capacity to survive and establish in the surrounding environment is unknown. The extreme temperatures and lack of moisture in Antarctica are generally considered to be one of the primary barriers to invasive species establishment in the region; however, research suggests that some species of spiders, such as redbacks (Latrodectus hasselti), show little significant response to exposure to low temperatures, with the exception of some individuals moving more slowly (Smith et al. 2015). Further, populations of invasive Australian redbacks in Japan demonstrated higher fecundity at low temperatures than native populations, possibly as a result of selective adaptation during the invasion process or rapid adaptation in phenology (Mowery et al. 2022). Several species of wolf spider persist as predators in the Arctic tundra and so it is possible that some species of Araneae possess the necessary adaptations to survive in Antarctica's harsh conditions. This is particularly true for sub-Antarctic islands where native spider species occur (Pugh 2004) and the Antarctic Peninsula as the climate warms, moisture availability increases and ice-free areas increase (Lee et al. 2017, 2022a). Indeed, climate matching may precondition non-native spiders to successfully establish (Kobelt and Nentwig 2008), making cool-climate species from Tasmania a greater risk under climate-warming

scenarios. Further research, such as species distribution modelling, may provide more insight into the establishment risk of commonly transferred non-native invertebrates in east Antarctica.

Given the ongoing risk of inadvertent transfer of non-native species through operational activities in Antarctica, we harnessed reports of BRM detections made in the AAD environmental incident database since 2004 to create a consequence table that can be applied by operational staff on-ground to improve biosecurity procedures and resource use. Our study has highlighted that cargo and consumable supplies act as a conduit for a large proportion of BRM detected in the AAD's operations and we, therefore, suggest that these areas continue to be subject to a high degree of scrutiny for biosecurity personnel and decision-makers. We also found that the majority of BRM reports are made at Antarctic stations, indicating that most non-native species are either not detected until after cargo, supplies and luggage are unpacked and de-consolidated upon arrival, reduced access to and, thus, inspection of, cargo during voyages, or that BRM detected on transport vessels is not reported as vigilantly as those found within stations. Indeed, general biosecurity procedures on-board ships are not as strict as those implemented at the cargo facility or on station due to logistical constraints, despite the presence of lights, warmth and food sources to attract invertebrates. The proposed consequence table will enable rapid responses to detections during de-consolidation, but will also determine when ongoing monitoring is required to reduce the risk of additional BRM remaining undetected. It is recommended that the consequence table be reviewed decadally to keep pace with changes in processes that are expected to increase the risk of non-native species having a biological impact on Antarctica's ecosystems, including climate change, growing human activity, new infrastructure and increased transportation to and within the Antarctic Region (Chown et al. 2012; Duffy et al. 2017; Galera et al. 2018; Lee et al. 2022b). The consequence table has been designed to be easily implementable by end users of any level of expertise, enabling the implementation of a rapid response. Given the sensitivity of the Antarctic environment to non-native species, it is advised that staff continue to opt for management actions (e.g. thorough surveillance, deep cleaning, insecticide treatment and incineration of infested supplies (see Bergstrom et al. (2018)) associated with the highest level of perceived consequence, in line with the precautionary principle (Peel 2005; Peterson 2006).

We have shown that, while policy instruments for the prevention of BRM introductions into the Antarctic Region continue to be developed and enhanced, the risk of introductions is ongoing. While we detected no increase in reported BRM detections in the AAD's operations in recent years, the relatively small dataset and short time period of the study makes significant trends difficult to determine. It is clear, however, that the transport of non-native species to Antarctica remains a risk as anthropogenic activities continue in the region. The ongoing surveillance and documentation of BRM and continual improvement of BRM management and response by the AAP demonstrates its commitment to environmental stewardship and contributes to its status as a leading National Operating Party in the Antarctic biosecurity space. To continue to support best-practice biosecurity, we highlight areas of concern, including the continued transport of live spiders, the ongoing potential for cargo to harbour BRM, particularly during ship voyages to Antarctica where biosecurity scrutiny is lower than at departure and arrival, and the need for ongoing training and investment and support for expeditioners in the reporting of BRM detections. We present a consequence table for operational staff and decision-makers to apply on-ground, enabling rapid responses to BRM detections with an appropriate allocation of resources.

Recommendations for decision-makers

Based on the outcomes of this study, we make the following recommendations to national operators in Antarctica:

- Implement a comprehensive, mandatory biosecurity reporting system at all stages of the transport pathway to Antarctica (as is currently implemented by the AAP and AAD) and provide appropriate training on BRM detection, identification and reporting for staff, crew and expeditioners. Given the collaborative nature of the Antarctic Treaty System, the establishment of a shared reporting platform for all national operators in the future would encourage transparency, as well as facilitate the sharing of management strategies and training information between parties.
- Monitor non-native species entrainments for live transport of high-risk taxa (e.g. spiders) and revise biosecurity protocols where necessary.
- Implement a rapid response framework that will enable consistent, appropriate responses to detections of biosecurity risk material without compromising operations.

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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: ZTC, IRO. Data curation: IRO. Formal analysis: IRO, MJH. Methodology: IRO. Supervision: PC, JS. Validation: KL, MJH. Visualization: IRO. Writing - original draft: IRO. Writing - review and editing: MJH, PC, KL, ZTC, JS.

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Data availability

De-identified data used in this study can be accessed via the Australian Antarctic Data Centre (http://dx.doi.org/doi:10.26179/cdpm-g422).

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Supplementary material 1

Full summary of locations of biosecurity risk material detections by taxonomic order

Authors: Isabelle R. Onley, Melissa J. Houghton, Kirsten Leggett, Phill Cassey, Zachary T. Carter, Justine Shaw

Data type: docx

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Methods

Design of an eDNA sampling method for detection of an endophagous forest pest

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NeoBiota

Abstract

Invasive wood-boring insects are a major economic and ecological concern worldwide as they impact native woody plant populations. These pest species are increasing in prevalence, with devastating impact, as global trade leads to higher rates of introduction and establishment. The emerald ash borer (Agrilus planipennis; EAB) is one such species, which has caused widespread damage across much of the United States and is now spreading across Europe. Non-indigenous woodborers such as EAB are difficult to detect at early stages of invasion, which is when management and eradication efforts are most effective and cost efficient. Environmental DNA (eDNA) surveys have demonstrated power in detecting invasive species when rare in the landscape due to their ability to detect trace amounts of DNA and identify to species. Here, we trialled a novel eDNA method for collecting environmental samples within host trees where invasive pest larvae are feeding, using EAB as a case study. We extracted tree cores approximately 1 cm in length using an increment hammer to assess detectability of eDNA from larvae feeding under the bark. In trees visibly infested with EAB, we observed a seasonal peak in EAB DNA detection probability (~ 64%; towards the end of the growing season), indicating a potential impact of ash tree phenology or EAB phenology on detection. When we trialled the method in a site with ash trees of low or uncertain EAB abundance, we did not record positive EAB eDNA detections. This outcome may have resulted from differing EAB phenology at the northern latitude of this survey site or because larval galleries were less numerous causing EAB DNA to be scarcer within the tree. Our results, however, provide preliminary evidence that increment hammer tree cores can be used to detect eDNA of EAB and, perhaps, other wood-boring pests. Further work is needed to clarify false negative survey detections at ash trees showing little to no signs or symptoms of infestation, as well as investigating the deposition, transport and persistence dynamics of EAB eDNA within trees.

Key words: *Agrilus planipennis*, forest management, early detection, emerald ash borer, environmental DNA, wood-boring insects

Introduction

Phloem- and wood-boring insects are the most economically costly amongst invasive forest pests globally, resulting in over \$2 billion in damage annually in the US alone (Aukema et al. 2011). They are also ecologically destructive, contributing to the functional extinction of formerly common tree species across several regions (D'Amato et al. 2023). Wood-boring insects feed and develop within their host trees, complicating early detection, eradication and suppression of invasive



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Copyright: © Kathleen E. Kyle et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). populations (Liebhold et al. 2012). Environmental DNA (eDNA) surveys are now commonly used to detect aquatic invasive species and their use to detect forest pest insects is an emerging, yet growing, field of research (Valentin et al. 2020; Kirtane et al. 2022). eDNA-based sampling methods frequently yield a substantial boost in species-specific survey detection probability over conventional methods (Jerde et al. 2011; Allen et al. 2021) and are increasingly considered as part of biosecurity surveillance programmes for this reason (Trujillo-Gonzalez et al. 2020, 2022). Development of an eDNA survey that improves detection capability for wood-boring insects would improve our ability to control and eradicate newly-established invasive populations (Liebhold et al. 2016). Here, we report on a novel eDNA methodology for detection of wood-boring insects that utilises tree cores taken from host species. We illustrate the technique by evaluating its performance at detecting the presence of emerald ash borer (*Agrilus planipennis*; EAB), a highly destructive invasive woodborer of ash trees (*Fraxinus* spp.).

EAB is the most economically costly invasive insect in the United States (Aukema et al. 2011) and it has recently become established and is spreading in Europe (Volkovitsh et al. 2021). First introduced to the US in the 1990s (Siegert et al. 2014), it went undetected for at least a decade as the damage it caused was originally misidentified (Cappaert et al. 2005). Methods of detecting EAB have improved since it was initially identified in the US. Visual surveys are commonly used to identify older, high-density EAB infestations by searching for signs and symptoms, such as ash canopy dieback, epicormic sprout development, presence of adult EAB emergence holes and woodpecker damage associated with foraging on late instar EAB larvae. Other survey tools, such as girdled 'trap trees' and baited artificial traps that rely on visual and chemical attractants, have been developed (McCullough and Poland 2017; Siegert et al. 2017). The lack of a strong long-range lure for EAB, however, has consistently led to low detection rates for artificial traps, which limits their utility for identifying and delineating EAB populations and, in turn, the success of management and eradication efforts (Siegert et al. 2014; Tobin et al. 2014). Girdled 'trap trees' remain a highly effective tool for detecting low-density EAB infestations, but have the disadvantages of having to sacrifice an ash tree, lags in detection until sampling may occur at the end of the season and the need for additional resources and skills to fell and sample for EAB larvae feeding under the bark (Siegert et al. 2017). Thus, there remains a need for additional survey tools that are powerful, cheap to deploy and can be used to survey large areas for low-density EAB populations.

We posit that DNA from EAB larvae feeding within ash trees may be accessed via the collection and processing of 1 cm tree cores (Fig. 1). Our expectation stems from observations of other wood-boring and chewing insects leaving detectable amounts of their DNA on the surface of plants they utilise for food, including within the internal tunnels they create (Pirtle et al. 2021; Taddei et al. 2021; Sickel et. al. 2023). We thus expect that EAB DNA can be captured in tree cores if taken within or very close to galleries (Fig. 1). Further, if DNA from feeding EAB larvae becomes entrained in an ash tree's transport networks, it could move within the tree just as other large organic molecules and metals do (Falcone and Cooks 2016; Alvarez-Fernandez et al. 2020). If so, we suggest that EAB larval DNA may be detected in tree cores taken at distances away from galleries (Fig. 1). Finally, if EAB DNA is moving within the transport tissues (i.e. phloem and active xylem) of an ash tree, we hypothesise that the flow of DNA will follow the seasonal movement of water and nutrients within the tree; moving up towards the leaves in the spring



Figure 1. Schematic of our expectation that larval emerald ash borers (*Agrilus planipennis*; EAB) deposit sufficient DNA as they develop under the bark to be detectable in tree cores from within or nearby feeding galleries. EAB DNA deposited in feeding galleries may also become incorporated into the tree's transport tissues surrounding the galleries and, if so, cores collected at some presently unknown distance from a gallery may correspondingly contain detectable EAB DNA. No matter the source, we expect the availability of EAB DNA from tree cores to be highly seasonal and peak either when larvae are most actively feeding or when the host tree moves large quantities of nutrients and water to the leaves or roots – shown here by downward arrows – in the transport tissues (beginning and end of the growing season, respectively) or a combination of the two. In the present study, core samples were extracted from sample trees at breast height (1.37 m) above ground level.

and down towards the roots in the autumn for trees of temperate biomes such as ash (Mariën et al. 2019; Fig. 1). To test these hypotheses, we extracted cores from EAB-infested ash trees over the course of a single growing season and developed a quantitative polymerase chain reaction (qPCR) workflow to detect the presence of EAB DNA within these cores. We also challenged our method by collecting ash tree cores within trees that had no or low visible EAB damage and, thus, likely fewer EAB larvae and subsequently less DNA deposition.

Methods

Species-specific EAB assay design

To test our hypotheses, we first had to design a qPCR assay that could detect trace amounts of DNA from an environmental source and accurately assign species identity to EAB and no other co-occurring species. This process involves, first, identifying a candidate species-specific amplicon and then measuring assay sensitivity and specificity (Bustin and Huggett 2017). To obtain samples representative of the genetic diversity of EAB populations in North America, we sequenced the COI region from 12 EAB specimens from the epicentre of the invasion in Michigan, courtesy of the USDA APHIS PPQ, EAB Biocontrol Rearing Facility in Brighton, MI. We also collected EAB specimens from infested ash trees in Rhode Island (1), Vermont (4) and New Jersey (4); these individuals aided capture of genetic diversity across the north-eastern region of the invasive range in the US. We used a combination of adult and larval specimens, identified by specialists and preserved either via desiccation or in ethanol, respectively. We used forceps flame sterilised with 100% ethanol (EtOH) to extract one hind leg from each adult specimen along with connected muscular tissue or we cut ~ 1/3 of a larval body and placed these tissues into sterile 0.2 ml tubes. We then extracted DNA from these tissue samples using the HotSHOT method (Johnson et al. 2015) and stored extracted DNA at -20 °C until subsequent processing.

We amplified part of the COI mitochondrial DNA region via polymerase chain reaction (PCR) using primers LepF1 and LepR2 (Herbert et al. 2004). Optimised PCR cycling parameters consisted of an initial hold at 96 °C for 10 min followed by 50 cycles of denaturation at 96 °C for 30 seconds, annealing at 44 °C for 40 seconds and extension at 72 °C for 45 seconds, with a final extension step at 72 °C for 5 minutes. DNA was amplified in 20 µl reactions consisting of 1X PCR buffer II, 2.75 mM MgCl₂, 250 pM each dNTP, 200 nM of each primer and 1.5U of Taq Polymerase Gold. We ran all PCRs on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, CA) and visualised reactions in 1% agarose gel with gel red dye. Successful reactions yielded amplicons roughly 300 bp in size that were cleaned using the ExoSAP-IT enzyme (Carlsbad, CA). Then, approximately 10 ng of cleaned template DNA was mixed with 25 pmoles of each primer and sent for bidirectional Sanger sequencing (GenScript, Piscataway NJ). Using Geneious Prime (version 2021.0.1), we trimmed the sequences, combined them with 35 additional EAB sequences from GenBank (Accession numbers provided in Appendix 1) and aligned them to identify a consensus sequence that was conserved across individuals of the species. We then used Primer Express (version 3.0.1) to identify a TaqMan qPCR primer and probe set optimal for amplifying a segment of this conserved region, hereafter referred to as "EAB COI assay".

All qPCR reactions consisted of 500 nM each primer, 250 nM probe, $1 \times TaqMan^{\circ}$ Environmental Mastermix II with no UNG and 2 µl DNA. The optimised reaction protocol included an initial denaturing step of 96 °C for 10 min, followed by 45 cycles of denaturation at 96 °C for 15 s and annealing and extension at 60 °C for 1 min. All reactions were run on an Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA). Each sample was tested for the presence of EAB eDNA in triplicate and considered positive if at least one of three technical replicates successfully amplified.

High-density EAB infestation: Tree core sampling through time

To investigate whether larval EAB DNA was present in tissues of ash trees, we took core samples (containing cambium, phloem and xylem) from green ash (Fraxinus pennsylvanica) throughout the 2021 growing season at two sites in New Brunswick, New Jersey, USA. We sampled 7-13 trees per day on 13 dates between 21 May and 15 October (Table 1). We chose initial sampling dates to coarsely coincide with growing degree day (GDD) accumulation thresholds for major EAB life history events. Adult emergence begins around 450 GDD - base 50 °F (10 °C) - and peaks around 1100 GDD (Siegert et al. 2015). As such, we estimated that our first sampling event, in May, was during the EAB pupal period; our second event, in June, coincided with peak EAB adult emergence; and our last weeks of sampling, from August through October, were when larvae had hatched from eggs, burrowed into trees and begun feeding. We elected to perform weekly sampling during this later time-frame assuming that EAB DNA may be deposited at higher rates by larvae feeding within galleries. In addition, EAB DNA could be transported within vascular tissues to the base of the trees during autumn senescence (Alvarez-Fernandez et al. 2014; Falcone and Cooks 2016).

Core samples were taken at breast height (~ 1.37 m above the ground) using a Haglöf 2.5 cm (1-inch) increment hammer at ash trees ranging from ~ 15 to 90 cm diameter at breast height (dbh). All trees showed visible signs of EAB colonisation (e.g., canopy dieback, epicormic sprouting); however, no bark splits or adult exit holes were visibly evident within 1 m of locations where we extracted tree cores. We were not able to strip bark from our sample trees after core extractions as they were on private property and we also wished to resample the same trees. Thus, we cannot be sure how close core samples were to larval feeding galleries or pupation chambers. Before taking each sample, we flame-sterilised the cutting tube of the increment hammer using 100% EtOH to remove any surface DNA between sample collections. At each tree, we took two 1 cm core samples (one each from north

Calendar Date (2021)	Ordinal Date	GDD Accumulation Base 50 °F (10 °C)	Sample Size (n)
21 May	141	366	20
30 June	181	1185	20
6 Aug	218	2105	14
13 Aug	225	2307	24
20 Aug	232	2504	22
27 Aug	239	2702	22
3 Sept	246	2853	16
10 Sept	253	2990	26
17 Sept	260	3148	26
24 Sept	267	3284	26
1 Oct	274	3367	26
8 Oct	281	3474	20
15 Oct	288	3584	20

Table 1. New Jersey core collection dates along with ordinal date and accumulated growing degree days and sample size for each sampling date.

and south aspects). We placed the core samples into tissue disruption tubes for later DNA extraction. In total, we took 282 cores from 21 ash trees. On each sampling date, we also extracted 1-cm cores (n = 2 or 4, depending on the number of sample sites) from nearby oak trees (*Quercus* spp.) expecting that these could serve as 'field negative control' cores where we would not expect EAB DNA to be present.

In the laboratory, we extracted DNA from core samples using the DNeasy Plant Pro Kit (Qiagen) following manufacturer's protocols and tested all samples via qPCR with our EAB COI assay as described above. Each DNA extraction and qPCR run included negative controls to ensure no in-lab contamination occurred.

Low-density EAB infestation: Tree core sampling across varying infestation levels

On 22–23 September 2021 (ordinal dates 265–266), we collected tree cores from white ash trees (*Fraxinus americana*) in Loudon, New Hampshire, USA to explore the ability of the method to detect EAB when present at lower densities. This EAB infestation was much less advanced than at our New Jersey sampling sites. Host trees at our New Hampshire sites exhibited a wide array of decline, characterised on a spectrum from no visible signs of infestation ('no damage') to minimal signs of infestation ('light damage') to a small degree of dieback and epicormic sprouting ('moderate damage'). Samples were taken using the same tree core methods as described above. Accumulated growing degree days at this site during the two-day sampling effort were 2224 and 2245. We collected samples from a total of 30 trees, 10 each from three tree damage categories: no damage, light damage and moderate damage. We extracted four tree cores, one from each cardinal direction, from each tree to increase probability of EAB detection. In total, 120 cores were taken from ash trees. Negative control cores were also taken from nearby birch trees (*Betula* spp.) to confirm complete decontamination of the increment hammer between samples.

Statistical analysis: Tree core sampling through time

We fitted a Bayesian generalised additive model (GAM) with a Bernoulli error distribution and a logit link function to describe the phenology of EAB eDNA detection over the course of the 2021 sampling season in New Jersey, as well as to assess the effects of covariates on detection rates (GDD accumulation and side of tree). Additionally, due to potential contamination (i.e. amplification of small quantities of EAB DNA) found in some negative control samples (see Results), we also conducted a parallel and more conservative set of analyses that were identical to those described above, but that only treated samples with at least two of the three qPCR technical replicates amplifying to be true detections (see Appendix 2). As it was visually evident that all 21 trees sampled were infested with EAB (see above), all detections and non-detections were assumed to provide information for the probability of detection given presence. The dependent variable was eDNA detection (1) or non-detection (0), based on the qPCR assay results for each sample (i.e. '1' if any of the qPCR technical replicates for a sample amplified EAB DNA, '0' otherwise). Independent variables in the model included a random effect of sample tree and two fixed effects: a binary variable indicating side of tree (north or south) and a continuous variable for the number of accumulated growing degree days (GDD) on the date of sampling. GDD data were obtained from the Cornell

Network for Environment and Weather Applications (NEWA). We used vague priors on all parameters and ran four chains of 4000 iterations each, including 2000 discarded warm-up iterations. Convergence was assessed using Gelman-Rubin statistics (rhat < 1.1). We compared models with different combinations of independent variables, based on leave-one-out information criterion (LOOIC).

We used the modelled detection probabilities from the GAM (i.e. the posterior distributions) to conduct an additional analysis to estimate how many core samples would be required to detect EAB in an infested tree with 95% certainty. To do this, we used the formula $p^* = 1 - (1-p)^n$, where p^* is the probability of obtaining at least one positive core sample, p is the modelled per-sample detection probability and n is the number of core samples taken. We set p^* equal to 0.95 and solved for n to estimate the number of cores required for each sampling event to have a 95% probability of detecting EAB presence.

Results

Species-specific EAB assay design

Based on the sequences we generated and those publicly available on GenBank, we designed primers EAB_COI-F (TTCGAGCAGAATTAGGAAATCCA) and EAB_COI-R (AAGCATGAGCAGTAACAATAACATTATAGA) and probe EAB_COI-Probe (CATTAATTGGCAATGACC), which target a 78 bp fragment within the COI mtDNA region of EAB. Our specificity testing indicated that our assay was specific to our target species (see Appendix 1). Based on these tests, we assume that any core samples that amplified with the EAB COI assay indicate EAB individuals living within sampled trees.

High-density EAB infestation: Tree core sampling through time

Of the 282 tree cores collected over the 2021 growing season, 120 tested positive for EAB DNA. Comparison of the GAMs explaining EAB eDNA detection probability indicated that the model including only the random effect of tree and the fixed effect of GDD was most parsimonious, as indicated by LOOIC comparison of models (Table 2; Fig. 2). The side of the tree where core samples were taken (north vs. south) showed little influence on detection probability ($\beta = 0.07, 95\%$ CI = [-0.46, 0.58] from the model including this variable plus GDD). The model revealed a hump-shaped temporal pattern in the proportion of samples testing positive for

Table 2. Comparison of Bayesian generalised additive models (GAMs) describing the phenology of emerald ash borer tree core eDNA detection probability, based on leave-one-out information criterion (LOOIC).

Model	ΔELPD ^a	ΔELPD SE	ELPD	LOOIC
Tree + GDD	0.0	0.0	-176.4	352.8
Tree + GDD + direction	-0.8	0.3	-177.2	354.4
Tree	-13.2	5.8	-189.6	379.2
Tree + direction	-14.4	5.8	-190.8	381.6

^a Difference in ELPD (expected log pointwise predictive density) between the model and the model with the highest ELPD; higher values represent more parsimonious models.



Figure 2. Top: probability of detecting eDNA from emerald ash borer (*Agrilus planipennis*; EAB) using the tree core sampling method at sites in New Jersey, USA over time. Bottom: the number of ash tree core samples needed per tree to ensure 95% confidence in detection of EAB larval presence within a tree. Time is represented as growing degree day (GDD) accumulation, base 50 °F (10 °C). Black lines represent the posterior medians from a Bayesian general additive model (GAM); grey shading indicates 80% and 95% credible intervals. Black circles show the proportion of samples with detections on each sampling date calculated from the raw data.

EAB DNA as growing degree days accumulated, with peak detection probability occurring at ~ 3000 GDD (Fig. 2). After this peak, the proportion of positive core samples dropped off, indicating that, at least in New Jersey, a sampling date around mid-September is optimal to collect ash tree core samples to detect the presence of EAB larvae within an infested ash tree. The temporal trends observed in detection probability were clearly reflected in the estimates of the number of cores required to obtain an EAB detection with 95% certainty (Fig. 2). This analysis revealed that, at peak detection probability (~ 3000 GDD), roughly 2.5 core samples per tree would be required to detect EAB larvae within a tree with 95% confidence (Fig. 2).

None of our laboratory negative controls revealed contamination. However, three of our 30 'negative control' *Quercus* tree cores returned weak positive EAB detections. These samples had cycle threshold (Ct) values of 39–41 and all had only one of three technical replicates amplify. The parallel analysis of detection probability that used stricter guidelines for declaring samples as 'positive' for EAB DNA revealed very similar phenological patterns (Appendix 2: Fig. A2).

Low-density EAB infestation: Tree core sampling across varying infestation levels

Of the 120 core samples we took from ash trees at the lower EAB density site in New Hampshire, we recorded no positive qPCR detections of EAB. All negative controls also returned negative qPCR results. This sampling effort took place at ~ 2200 accumulated GDD, potentially missing the point when peak detection was witnessed during our high-density sampling season that took place at a more southern latitude.

Discussion

Our results indicate that DNA deposited by EAB larvae can be recovered from ash tree cores using a standard increment hammer and confirmed via species-specific qPCR detection. We also show that there may be strong seasonality to this method of EAB detection. This seasonal pattern follows our hypothesis that, as they feed, EAB larvae deposit their DNA within galleries or it becomes incorporated within ash tree tissues. Further, the peak in seasonal eDNA detection probability of larvae was about three months later than the peak trap catch dates in conventional survey methods for capturing adults (Tobin et al. 2021). The optimal timing for conventional trapping methods is considerably earlier in the growing season (~ 1000 accumulated GDD, which corresponds to late June in New Jersey; Tobin et al. (2021)). This result suggests the potential utility of eDNA for survey season extension. Our results represent a novel methodology for detecting the presence of wood-boring insect larvae within their host trees and may prove particularly useful for detection of invasive insects within forested landscapes or urban environments more broadly. However, our results were less encouraging regarding the detection of EAB when present within ash trees at low enough abundance that the trees exhibited little visual evidence of infestation. These mixed results suggest several avenues for continued research, which we detail below.

Phenology is an important consideration in any population assessment method (e.g. Kean and Stringer (2019)) and our results showed that this concept also applies to eDNA detection via tree core surveys. We show that, if cores are taken at the optimal time of year, then eDNA survey detection rates can be quite high: ~ 64% per core sample for EAB at their peak; or, requiring only 2–3 cores per infested tree to detect EAB with 95% confidence. Conversely, like conventional methods, if samples are taken at the wrong time of year, detection rates can be very low or require a prohibitively large number of samples to confirm EAB larval presence. It is unknown, without a head-to-head comparison, whether eDNA detection rates are higher or lower than conventional tools and how they perform relative to one another when EAB are present at very low densities. However, the relative immediacy and ease of collecting eDNA core samples (e.g. few to no revisits or tree girdling and debarking required) alone make this a potentially attractive survey tool. Our method provides logistic ease that conventional approaches may not. For example, one person in the field can

easily take tree cores and store them for later processing, moving and sampling over a wide spatial extent in a single day. Samples can then be processed quickly in the lab using our qPCR approach or our methods can be adapted for use in other molecular workflows that vary in their immediacy and sensitivity (e.g. field-based qPCR, LAMP, digital droplet PCR). However, the relative feasibility of each workflow requires independent evaluation. For instance, the extraction of DNA from wood is not as straightforward as it is from frass or tissue and may be difficult to accomplish in the field, potentially increasing turnaround time from sample collection to result. This is certainly another research front that needs exploring for this method to be adopted on a wide scale. Though given how rapidly eDNA technologies are presently advancing, it is likely that improved methods for DNA extraction and more sensitive molecular techniques will aid to expedite the vetting process of this method.

The exact causes for the seasonality we observed in tree core eDNA detection rates are unknown. If EAB eDNA is indeed transported throughout the tree along with water and nutrients, the role of tree physiology becomes a key consideration in survey design. The ecophysiology of sap flow in trees has a long research history (e.g. Zimmermann (1957)) and could aid in optimising timing for when to conduct tree core eDNA surveys and possibly help identify within-tree sampling locations across seasons (e.g. leaves, twigs, trunk, roots). In contrast, the dynamics of DNA deposition by endophagous insects within host plants is a new field of study (e.g. Pirtle et al. (2021)). While there is a growing research base that has investigated the movement and degradation of eDNA in aquatic systems (Strickler et al. 2015) and on surfaces within terrestrial systems (Valentin et al. 2021), very little is known about how long arthropod DNA may persist within larval galleries or transport tissues of trees. This DNA is protected from UV degradation, which should allow it to persist in a detectable state for long periods of time (Valentin et al. 2021). However, it may also experience a suite of thermal, chemical and biological interactions within trees and their tissues that may degrade the DNA, limiting the time-frame over which it can be detected by a molecular assay. As with other eDNA techniques, an in-depth understanding on the 'ecology' of arthropod eDNA under bark and within transport tissues of trees is needed before our tree core approach can be widely adopted for use in invasive species management.

Nutrient flow within ash trees is restricted when larval EAB densities are high because larval galleries serve to interrupt phloem movement (Flower et al. 2018). This pattern suggests that the high eDNA detection rates found within highly-damaged trees may only reflect a 'pooling' of EAB DNA in tree tissues near larval galleries. Our tree cores may be capturing EAB DNA from these pools and/or from the inner surfaces of larval tunnels, which are rich in DNA from frass and chewing of EAB larvae (Pirtle et al. 2021; Taddei et al. 2021). When trees are healthier, any deposited EAB DNA may be transported over much more of the tree making it less likely to be captured when extracting only a small number of tree cores. Similarly, trees with low densities of EAB larvae will have fewer galleries making it less likely that any core sample taken will include tissues from gallery tunnels. These possibilities may explain, in part, our failure to detect EAB DNA within cores taken from trees with lower visual evidence of damage. It is also possible that the timing of our low-density sampling effort was offset enough from the autumnal senescence of these more northern ash trees that we simply missed the detection peak there. We witnessed overall much lower GDD accumulation at our low-density sampling location (~ 2200 GDD) than when the probability of detection peaked in our high-density study (~ 3000 GDD).

However, there is also a suite of other factors that impact the onset of autumnal senescence aside from GDD accumulation that could point towards the differing 'peak' detection timings at these different latitudes (Gill et al. 2015).

The extent to which the phenology of tree core eDNA detection does not overlap with the phenology of detection from conventional methods suggests an opportunity to use this new tool, once fully vetted, to extend the window of EAB detection for delimiting surveys or landscape-scale surveillance. Additionally, this combination of eDNA and conventional trapping programmes may apply to invasive wood-boring insects more generally, as most traps effectively catch the ephemeral flying adult stage of a target species, but are ineffective at detecting larval stages, which our eDNA tree core method could be well-suited to do. The eDNA tree core meth-od could also complement other forms of EAB surveys besides traps, for example, by concentrating tree core eDNA sampling activity on or near girdled 'trap trees', which represent the best-known attractant for the species (Siegert et al. 2017). For optimal management of EAB, having multiple well-tested tools that target a range of developmental stages is desirable – especially at invasion fronts such as Oregon, USA or western Europe – and also for other damaging wood-boring species (e.g. Asian long-horned beetle, *Anoplophora glabripennis*; Taddei et al. (2021)).

Our study introduces a promising and novel survey tool for detecting invasive wood-boring insects, along with proof-of-concept testing and insights into its performance. The key to realising if and when this method provides benefits to detecting EAB or other invasive wood-boring forest insects is: (1) executing an explicit test of the sampling effort necessary to detect EAB across different larval abundances; (2) a controlled repeat of our methods across a more inclusive array of EAB densities and latitudes to identify seasonal peaks and the degree to which these peaks diminish with lower EAB infestation levels; (3) identification of how much higher or lower detection probability of EAB is as a core sample is taken at varying distances away from larval feeding galleries and 4) a side by side comparison of landscape-level detection probability with the tree core method versus traditional trapping and detection techniques. It will only be possible for this tool to become operational after investigating these questions to better provide information about the power of the method as well as the potential shortcomings. Given the impacts of invasive wood-boring insects and the increasing number of invasions that are occurring globally (Aukema et al. 2011), having an effective and logistically simple sampling tool to increase detection probabilities for these species would contribute to preventing and mitigating the damage they cause to trees and forest ecosystems worldwide.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Data availability

The protocol for this newly-designed eDNA sampling method has been published on our lab environmental DNA website (https://sites.rutgers.edu/edna/). All sequences we generated during this study will be publicly available on GenBank (Accession numbers PP373086–PP373114).

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Appendix 1

EAB COI qPCR assay design, specificity and sensitivity testing

Methods

For the design of our EAB COI assay, we generated 21 sequences from specimens collected in the species' invasive range and combined with 35 sequences that were publicly available on GenBank. The GenBank accession numbers for those sequences are listed below:

AY756137, AY864194, DQ861319, DQ861320, GU013563, JF887747, KM845113, KT250461, KT250462, KT250467, KT250473, KT250476,

KT250479, KT250480, KT250487, KT250490, KT250504, KT250508, MF286180, MG477998, MH159080, MH159105, MN548248–MN548260.

We conducted *in silico* specificity testing on 10 closely-related sympatric species to ensure none would cross-amplify with the assay we designed. We also performed a nucleotide blast in GenBank to ensure no published sequences had high percent identity with our target amplicon sequence. Finally, we conducted *in vitro* specificity testing on seven co-occurring Coleoptera with specimens trapped together at EAB invasion sites in Connecticut.

We evaluated our assay's lower limits of detection (LOD) by creating an 8-level 10fold dilution series using genomic DNA (gDNA) extractions from EAB specimen legs with attached muscle tissue. We carried out qPCR analysis in 20 μ l reactions with 11 replicates of each concentration, which ranged from 1.6 ng to 1.6 fg as quantified using a Qubit Fluorometer (Invitrogen v. 2.0). We estimated the 95% limit of detection (LOD) of the assay by fitting a 3-parameter log-logistic dose–response curve to the resulting concentration and detection data following Klymus et al. (2020).

Results

Our *in silico* specificity test evaluated sequences of 10 relatives co-occurring in the US northeast and showed evidence that there are sufficient polymorphisms in the primer and probe regions such that they would not cross-amplify with our designed assay (Appendix 1: Fig. A1). For our *in vitro* specificity test, we ran extracted DNA from seven species (*Agrilus arcuatus, Agrilus bilineatus, Phaenops fulvoguttata, Spectralia gracilipes, Dicerca divaricata, Dicerca lurida* and *Actenodes acornis*) through our EAB COI qPCR assay and all samples returned negative amplification results. All species tested are order Coleoptera and family Busprestidae. Of all the species tested for assay specificity, the only one which is known to co-occur on ash (*Fraxinus* spp.) is *Agrilus subcinctus*.

The modelled 95% LOD of our EAB qPCR assay (Klymus et al. 2020) was 16 fg per reaction (95% CI = [6.25, 130]) assuming three technical replicates were performed. The lowest concentration of EAB genomic DNA detected from our serial dilution was 1.6 fg.



Figure A1. Based on published genetic data in conjunction with species sequences we generated, we created a *Geneious Prime* sequence alignment to analyse how many base pair polymorphisms existed between each of 10 co-occurring Buprestid species in the primer and probe regions we designed for our EAB COI qPCR assay (highlighted in grey). The sequences listed are consensus sequences based on a combination of those we generated ourselves as well as those accessed on GenBank.

We break down below the number and source of sequences used to generate these consensuses as follows:

Agrilus bilineatus: 1 sequence we generated + HQ582712, HQ582713, MF286166, MF805329, MH159018, MH159107

Agrilus arcuatus: MF286192, MF286193, MF805139, MF805156, MF805159, MF805179, MF805196, MF805245, MF805295

Chrysobothris femorata: 1 sequence we generated + JF888345, KR126263, KR481996
Agrilus quadriguttatus: 1 sequence we generated
Dicerca lurida: 2 sequences we generated + MG057907
Spectralia gracilipes: 1 sequence we generated + KM364375, KM847081
Agrilus subcinctus: 1 sequence we generated
Buprestis striata: KR482483
Brachys ovatus: HQ582477
Dicerca divaricata: AY165645, MG054990

Appendix 2

Accounting for potential contamination



Figure A2. This plot shows the same model output as in Fig. 2, but with detections defined more conservatively, as samples with at least two technical replicates successfully amplifying. Top: probability of detecting eDNA from EAB using the tree core sampling method at sites in New Jersey, USA over time. Bottom: the number of ash tree core samples needed per tree to ensure 95% confidence in detection of EAB larval presence within a tree. Time is represented as growing degree day accumulation, base 50 °F (10 °C). Black lines represent the posterior medians from a Bayesian general additive model; grey shading indicates 80% and 95% credible intervals. Black circles show the proportion of samples with detections on each sampling date calculated from the raw data. The GAM model was fitted and used default vague priors in accordance with Bürkner (2017).



Research Article

No evidence for pronounced mate-finding Allee effects in the emerald ash borer (*Agrilus planipennis* Fairmaire)

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Abstract

Allee effects are density-dependent barriers that can impact species establishment and population growth, such as through reduced mating success at low population densities. The emerald ash borer, *Agrilus planipennis* Fairmaire, has been extremely successful at rapidly expanding its North American range. The impact of mate-finding Allee effects (an important type of component Allee effect) early in the invasion period of the emerald ash borer remains unknown. We measured mating success in females as a function of beetle abundance in Halifax, Canada, where the emerald ash borer was recently discovered, and in Connecticut USA, where it has been established for over a decade. We measured relative population abundance and sampled beetles using different strategies. In Halifax, we placed clusters of prism traps along an invasion gradient of emerald ash borer abundance, and in Connecticut, we collected beetles from foraging *Cerceris fumipennis* females. We dissected female reproductive tracts to measure mating success. We fit a linear regression to the mating success of females as a function of beetle abundance. We found that emerald ash borer did not present a pronounced mate-finding Allee effect as there was no positive relationship between female mating success and abundance. Lack of pronounced component Allee effects that impede population growth may explain rapid range expansion in species that are highly invasive, such as the emerald ash borer.

Key words: Allee effects, emerald ash borer, invasion biology, invasion dynamics, mating success, population ecology



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Introduction

Introduced species must overcome several ecological barriers before establishment and population growth can occur. Such barriers include Allee effects, density-dependent mechanisms that prevent low-density populations from increasing (Allee 1938; Taylor and Hastings 2005; Liebhold and Tobin 2006; Gertzen et al. 2011). Allee effects often occur in introduced species because populations are small, leading to difficulty finding mates, inbreeding depression, high predation, or difficulty acquiring food (Courchamp et al. 1999, 2008; Kramer et al. 2009).

An introduced species that has caused widespread ecological and economic damage throughout North America in a relatively short period is the emerald ash borer (hereafter referred to as EAB), *Agrilus plannipennis* Fairmaire (Coleoptera, Buprestidae). It has been introduced from eastern Asia to North America and

eastern Europe (Cappaert et al. 2005; Valenta et al. 2017). In North America, it was first discovered in Detroit, MI, in 2002 (Haack et al. 2002), and has since spread rapidly throughout the eastern USA and Canada.

The rapid expansion of EAB in its North American range can be attributed to the movement of ash materials such as saplings, wood chips, and untreated firewood (Cappaert et al. 2005). Human transport of these products has led to the introduction of many satellite EAB populations across both planted and natural ash ranges in North America (Muirhead et al. 2006). EAB has infested more than 1,400 counties in Canada and the United States (Emerald Ash Borer Network 2022; United States Department of Agriculture 2023) and has caused substantial mortality to ash trees in North America (Herms and McCullough 2013).

Population modelling has shown that EAB could be managed if invading populations were under a strong demographic Allee effect (McDermott and Finnoff 2016) - that is, if there were a threshold population density below which low mating success leads to a negative population growth rate (Fauvergue 2013). Each long-distance movement would subject the insect to the ecological pressures of establishing new populations at low population densities. However, incipient EAB populations are difficult to monitor accurately, affecting the ability to manage populations early in the infestation (Siegert et al. 2014; Ryall 2015). Consequently, the importance of Allee effects during the establishment period of EAB is largely unknown. Specifically, empirical studies on density-dependent mate-finding in EAB remain limited (McDermott and Finnoff 2016). Mating failure has been suggested as a mechanism (a "component Alee effect") for negative population growth rates at low population densities due to the inability of males and females to locate each other when sparsely distributed on the landscape (Fauvergue 2013). Pronounced mate-finding Allee effects are known for at least some invasive forest pests, including brown spruce long-horn beetle Tetropium fuscum Fabricius (Coleoptera, Cerambycidae) (Rhainds et al. 2015) and the spongy moth Lymantria dispar dispar Linnaeus (Lepidoptera: Lymantridiae) (Sharov et al. 1995; Contarini et al. 2009). Whether a similar effect exists for EAB is thus an evident and important question.

We examined the mating success of EAB as a function of beetle abundance in two distinct geographical locations. First, we studied a recently established satellite population in Atlantic Canada. In 2018, EAB were detected in a city park in the northeastern edge of the Halifax Regional Municipality of Nova Scotia. We measured female EAB mating success as a function of male abundance in scattered ash tree clusters along the current EAB range in Halifax, collecting beetles in pheromone-baited traps. Second, we studied a population in Connecticut, USA, about 800 km SW of Halifax. In this case, we measured female EAB mating success in individuals collected as prey by a native wasp and used the proportion of EAB captured by wasps among all beetle prey as a proxy measurement of abundance (Rutledge 2023; Rutledge and Clark 2023). In each population, we hypothesized that if pronounced mate-failure Allee effects were present, there would be a positive relationship between female mating success and beetle abundance. In contrast, if female mating rates were similar across all populations with different abundances, it would suggest that female mating failure at low population densities may not be an important factor in EAB population establishment (Fauvergue 2013). We did not find evidence for a pronounced mate-finding Allee effect in EAB in either location. However, we cannot rule out mate-finding Allee effect occurring below the beetle abundances we observed, nor Allee effects due to other density-dependent factors such as predation.

Methods

Study system

EAB can be distinguished from native North American wood borers (Coleoptera, Buprestidae) by its metallic green coloration and metallic red on the dorsal portion of the abdomen (Wei et al. 2004; Volkovitsh et al. 2019). Adults lay eggs in bark crevices of ash trees in the summer. Larvae emerge and feed on the phloem tissue beneath the bark, causing significant damage to the conductive tissues of the tree (Cappaert et al. 2005). EAB overwinter as late larvae or pupae and emerge from the bark the following summer (Bauer et al. 2004; Poland et al. 2011) although under conditions of high tree vigour or limited growing degree days, it can take individuals two years to complete their lifecycle (Wang et al. 2010). Peak adult emergence occurs at 450–500 °C (DD_{10C}) (above 10 degrees) and sharply ends at 833 (DD_{10C}) (Bauer et al. 2004; Tobin et al. 2021). Adult EAB will typically live for three to six weeks, when they feed on ash foliage, search for mates, and oviposit on ash trees, Fraxinus spp. (Bauer et al. 2004; Cappaert et al. 2005). Both male and female beetles can mate with several partners (Rutledge and Keena 2012). Sperm is transferred as a spermatophore to the female, and when the spermatophore dissolves, the sperm, bundled in groups of approximately 50 sperm in hyaline sheaths, are transported into the spermatheca bulb (Rutledge and Keena 2019) and can be observed by dissection and microscopic examination. The presence or absence of sperm in the spermatheca indicates the mating status of a given female.

Site and sampling design: Nova Scotia

We conducted the Nova Scotia study in 2022 using urban street trees and parks in the Halifax regional municipality (44°40'N, 63°36'W). The Bedford Basin, a large, enclosed bay, bisects the Halifax regional municipality. EAB was first detected at Harry DeWolf Park in 2018, at the interior tip of this basin. Halifax is a mid-sized city with a population of approximately 440,000 people and an area of 5,500 km². Halifax manages approximately 49,000 public trees (City of Halifax 2020). The most common species in the Halifax urban forest are Norway maple (Acer platanoides Linnaeus), American elm (Ulmus americana Linnaeus), and red maple (A. rubrum Linnaeus), accounting for about 32% of all publicly surveyed trees (City of Halifax 2020). Ash trees (Fraxinus spp.) account for approximately 1,225 trees, or 2.5% of all publicly surveyed trees in Halifax (City of Halifax 2020). Green ash (F. pennsylvanica Marshall) is the most common ash species surveyed, accounting for approximately 65% of all ash trees in Halifax. Nova Scotia is within the Acadian Forest, dominated by red spruce, balsam fir, yellow birch, sugar maple, eastern hemlock, eastern white pine, and red oak (Neily et al. 2017). Ash trees sparsely populate forested regions outside of Halifax, with highest populations (18%) in the southwestern portion of the province (Beaudoin et al. 2014). While EAB may have a preference for green ash, the preference does not appear to be strong (Anulewicz et al. 2008; Pureswaran and Poland 2009). Since its discovery in Nova Scotia, the provincial government has monitored EAB populations using green prism traps. Street trees were not treated with insecticides, enabling EAB to infest trees unhindered. In 2021, parasitoid wasp releases began but their range was limited to a few hundred meters from the release site, outside of our sampling locations (Cory Hughes, Natural Resources Canada, Pers. Comm., 2022).

To measure EAB populations, we arranged green (Andermatt Canada Inc., Fredericton, New Brunswick) and purple (WestGreen Global Technologies Inc., Langley, British Columbia) prism traps in clusters of at least four ash trees (Fig. 1). Each cluster had traps on four trees, with one green and one purple trap per tree. Green traps were baited with EAB pheromones (Silk et al. 2011) (3Z-lactone, 3 mg load rate, 50 μ g/day release rate, Andermatt Canada Inc., Fredericton, New Brunswick) and host kairomones (3Z-hexenol, 5 mL load rate, 50 mg/day release rate, Andermatt Canada Inc., Fredericton, New Brunswick) to increase trap catch. The attraction radius of traps baited with these pheromones is effective at approximately 28 metres from the pheromone source and as distance increases to 70 metres, becomes less effective (Wittman et al. 2021).

We chose cluster locations based on the availability ash trees on public land and at increasing distances from the initial infestation point, which in other studies has been shown to correlate with decreasing populations sizes (Mercader et al. 2009). Clusters were at least 350 meters apart to minimize trap interference (Wittman et al. 2021). Trees within clusters were at most 150 meters apart. We placed a green prism trap near the upper crown and a purple prism trap near the mid crown. Purple prism traps are reported to attract females as they resemble oviposition sites on tree bark (Francese et al. 2010). To increase trap catch, we placed traps on the south sides of the trees (Lyons et al. 2009) and placed two dead female EAB



Figure 1. Emerald ash borer collection in the Halifax Regional Municipality, Nova Scotia. Green circles represent sampled clusters of ash trees. Red star represents the location of the first ash borer detection in the Halifax Regional Municipality. Grey tree icons represent ash trees identified during street tree surveys.

as decoys in the upper left corner and near the center of each side of our traps (Domingue et al. 2015). We marked each decoy in white on the abdomen and head so that we didn't collect it, and we replaced as needed during weekly checks.

We measured degree days above 10 °C (DD₁₀) from a weather station monitored by the Government of Canada in Halifax to know when to deploy and finish trap collection. We deployed traps at the beginning of EAB flight on June 3^{rd} , 2022, at approximately 260 (DD_{10C}), and collected them on August 15th, at approximately 745 (DD_{10C}), when flight neared its end. We checked traps weekly, collected all the buprestid beetles into a cooler, then transferred them to a -18 °C freezer until dissection.

Site and sampling design: Connecticut

We conducted the Connecticut study in 2013–2016 and 2022–2023, using 48 sites across the state ranging from 41.43 to 41.88°N, and from-71.88–73.50°W. Emerald ash borer was first detected in New Haven County, Connecticut, USA in 2012 (Rutledge et al. 2013). Prior to, and for the first 2 years after, the invasion purple prism traps were widely distributed throughout the state and were responsible for detections in several counties. Simultaneously, a novel survey technique known as biosurveillance (Careless et al. 2014), was implemented, and after initial county detections had been made in over half of Connecticut's eight counties this has since become the sole continuing survey. Since then, EAB has spread to all corners of the state, and has been broadly destructive (Rutledge and Clark 2023).

Biosurveillance exploits the buprestid-hunting habit of a native wasp, Cerceris fumipennis (Hymenoptera, Crabronidae). These solitary ground-nesting wasps use paralyzed, adult buprestid beetles to provision their brood cells. Wasps live in aggregations in sandy areas, from which they set out to hunt in the canopy of the surrounding forest. They will target a wide range of genera within the Buprestidae, limited only by prey weight and size relative to the individual female wasp, phenology, and arboreal habit. More than 100 species of beetles have been recorded as prey of C. fumipennis (Rutledge et al. 2013; Careless et al. 2014). The proportion of individual species in the catch depends roughly on the proportion of those species in the surrounding 1.5 km of forest canopy (Rutledge 2023). This relationship is vividly illustrated during periods of unusual abundance of particular prey items, such as during the invasion of emerald ash borer (Rutledge and Clark 2023), the increased proportion of the hemlock borer (Phaenops fulvoguttata) a native-secondary pest of hemlock, suffering an outbreak of hemlock wooly adelgid in the mountains of western North Carolina (Swink et al. 2013), or the correlation between the proportion of conifers surrounding a colony, and the proportion of conifer feeding beetles captured by the wasps (Rutledge 2023). Thus, relative population densities of emerald ash borer can be a good measure for estimating the ratio of EAB to other buprestids captured by the wasps at the same time and place (Rutledge and Clark 2023).

We (CER and colleagues) have been conducting biosurveillance across the state since 2010, and all beetles collected have been identified by site and date. Since 2016, all EAB have been frozen and stored after capture, and thus were available for dissection. We selected female EAB for dissection from site/year combinations for which at least 30 beetles had been collected. This ensures that the estimate of relative abundance of EAB at that site was robust.

Identification and dissection of female EAB

For beetles captured in the prism traps, we cleaned glue residue from collected beetles using limonene. We identified and kept only EAB beetles. We identified females either based on their enlarged 1st abdominal segment and a lack of dense setae on the prosternum (Wang et al. 2010), or, for beetles not evidently expressing these characteristics, by the presence of an ovipositor on the last abdominal section. For beetles collected by biosurveillance, we chose well-preserved frozen specimens.

We dissected female reproductive systems by pulling the ovipositor with forceps, removing the reproductive tract, and mounting it onto a microscope slide. We cut the bulb of the spermatheca and gently squeezed it to push out any sperm. We stained the slide with Giemsa stain (Giemsa 1904) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to make sperm more visible. We assessed slides for sperm and scored beetles for the presence or absence of sperm. A female was considered successfully mated if we identified sperm in the reproductive tract (Rhainds 2010).

Statistical analysis

Estimating absolute abundance for EAB in an urban area with non-random ash tree distribution is difficult, and the appropriate sampling unit (such as area or number of ash trees) is not obvious. We chose instead to work with relative estimates of abundance: total male trap catch (per tree cluster) for Halifax and proportion of EAB collected from *Cerceris* wasps (per site) for Connecticut. For the remainder of the text, we use "abundance" to refer to this relative abundance estimate of beetles. Because we are using relative estimates, we cannot directly relate our Halifax and Connecticut abundance estimates. However, this does not matter, as we are only asking whether we can detect mating-abundance relationships within each region.

To test the hypothesis that mating success increases with beetle abundance, we fit weighted linear regressions of proportion mated females as a function of male abundance, separately for EAB collected from Halifax (by tree cluster) and Connecticut (by site). We weighted each data point by one divided by the square of the difference between the upper confidence interval (CI) and the lower confidence interval (CI) to account for the greater information contained in estimates from sites with more beetles. However, unweighted regressions supported identical conclusions (results not shown). An alternative analysis for Halifax with log transformation of male abundance yielded essentially identical results (not shown).

We used the Scipy (V.1.9.3) and Scikit Learn (V.1.0.2) packages in Python (V.3.9.16) to conduct all statistical analyses. We performed data visualization using the Matplotlib (V.3.6.2) and Seaborn (V.0.12.2) packages.

Results

Nova Scotia

We collected 1673 adult EAB (1329 males and 356 females) over the course of the study. Of the 356 females, 174 had sperm present in their spermatheca leading to a mean female mating success rate of 0.59 (± 0.072 SE). More than half

(983 individuals) of the beetles were collected from a site nearest the invasion epicentre. The number of males significantly predicted the number of females collected within clusters ($r^2=0.98$, $p=2.5e^{-14}$). For every female EAB collected in traps approximately 3.7 males were collected. Male abundance was not a good predictor of female mating success ($r^2=0.015$, p=0.63, 95% CI slope = [-1.2 e-03, 7.7 e-04], Fig. 2). Because the confidence interval includes some weakly positive slopes, we cannot rule out a subtle mate-finding Allee effect, although we can rule out a strong one (that is, more positive slopes fall outside the 95% confidence interval).



Figure 2. Female mating success as a function of relative male abundance. Collected from prism traps in Halifax, Nova Scotia, Canada, in 2022. Dots represent clusters of trees where beetles were trapped. Error bars represent 95% confidence intervals of the proportion of mated females at each location. The dotted red line represents the slope of the weighted linear regression (not significant). The dashed blue line represent 95% confidence intervals of the linear regression.

Connecticut

Relative abundance of EAB, as measured by proportion of EAB to other beetles collected, ranged from 0.020–0.935 with a median abundance total of 0.388 from 48 sites. The number of beetles collected at sites from which dissected beetles were chosen ranged from 31–288 with a median sample size of 63. We dissected 249 female EAB. Of the female EAB dissected, 168 had sperm present in their spermatheca indicating successful mating while 81 did not successfully mate as indicated by the lack of sperm.

Population abundance did not significantly predict female mating success ($r^2=0.000$, p=0.9, 95% CI slope = [-0.38, 0.34], Fig. 3). Because the confidence interval includes some weakly positive slopes, we cannot rule out a weak mate-finding Allee effect, although we can rule out a pronounced one (that is, more positive slopes fall outside the 95% confidence interval).



Figure 3. Female mating success as a function of relative EAB abundance. Collected from *Cerceris fumipennis* wasps in Connecticut, United States, from 2014–2016 and 2022–2023. Dots represent geographically distinct communities within Connecticut. Error bars represent 95% confidence intervals of the proportion of mated females at each location. The dotted red line represents the slope of the weighted linear regression (not significant). The dashed blue line represents 95% confidence intervals of the linear regression.

Discussion

Allee effects can have important influences on establishment success of non-native species, even determining whether populations continue to grow in the invaded habitat. Allee effects have been shown to influence population dynamics and suppress populations between outbreaks in both non-native forest insects such as the spongy moth (*Lymantria dispar dispar* Linnaeus) (Liebhold and Bascompte 2003) and native species such as spruce budworm (*Choristoneura fumiferana* Clemens) (Régnière et al. 2013) and mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Raffa and Berryman 1983).

Mate-finding Allee effects have been demonstrated in very few forest insect systems because it is typically difficult both to observe individuals at low densities and to determine mating status of those individuals (e.g., Kramer et al. 2009; Rhainds 2010). However, we were able to test for mate-finding Allee effects in two different regions, using two different approaches for estimating EAB abundance. In neither case did we find evidence for pronounced mate-finding (component) Allee effects. The proportion of female beetles that were successfully mated was consistent, despite varying male densities, suggesting that mate-finding failure may not occur in incipient populations of EAB. If an Allee threshold exists, the threshold occurs at even lower population densities than those we measured. Prism traps and biosurveillance have similar detection thresholds, and are generally used as early detection strategies for new infestations (Francese et al. 2013; Rutledge et al. 2013; Mccullough and Poland 2017) because they can be effective at beetle densities far too low for direct census (by branch dissections). It is certainly possible that mate-finding Allee effects kick in at densities too low even for these techniques to measure. Since this also means densities too low for infestations to be detected and studied, it would be a major challenge to document such extremely low-density Allee effects if they exist. Experimental manipulation of population densities could yield more power and better precision in estimating the mating-density relationship but would be both logistically and ethically challenging.

Studying low-density populations, ones that are frequently affected by component Allee effects, remains challenging due to the difficulty of finding small enough populations but detecting enough individuals to derive meaningful statistical conclusions (Liebhold and Bascompte 2003; Kramer et al. 2009). Once individuals are detected in high enough populations, they may no longer be affected by component Allee effects (McDonald 2004). Therefore, studying low-density populations can require significant amounts of resources to receive meaningful empirical measurements. We designed our study to be comprehensive in its ability to collect sufficient individuals by increasing sampling efforts in the Halifax study and collecting over a long time period in the Connecticut study.

Our results suggest that EAB is not subject to pronounced mate-finding (component) Allee effects through the range of abundances we studied. We found approximately half of collected EAB females were mated even at our lowest populations. This contrasts with some studies of other introduced species such as the brown spruce longhorn beetle (Rhainds et al. 2015) and spongy moth (Sharov et al. 1995; Johnson et al. 2006), in which mating success declines close to zero at low population density. In the case of the brown spruce longhorn beetle, invasion appears to have stalled at the current range edge, possibly due to mating failure (Rhainds et al. 2015; Anderson et al. 2022).

Even at our highest-abundance sites, a substantial fraction of females often remained unmated: mated fractions reached only 60–70% in Halifax, and 40–80% in Connecticut. For brown spruce longhorn beetle, Rhainds et al. (2015) found mating rates at around 80% in the largest populations, while for spongy moth, Sharov et al. (1995) found mating rates up to 100%. However, we used two sampling techniques that are designed to be very efficient at low beetle densities, and it's possible that sampling simply took females out of circulation early in their lives.

Mate-finding Allee effects in low-density populations generally exist when organisms are sparsely distributed in space and encounters among individuals are low. However, mate-finding difficulties can potentially be overcome if mate-finding is particularly efficient. This might be especially true in systems where mate-finding is multimodal, as it is for EAB. EAB uses multiple cues to identify host trees and find mates. Both males and females are attracted to volatiles of stressed ash trees, particularly 3Z-hexenol, a component of ash foliage (De Groot et al. 2008). In addition, they are visually attracted to wavelengths in the green range (530-540 nm) (Crook et al. 2009; Francese et al. 2010) as they spend a two-week maturation period feeding on foliage before mating. Females, at least, are also attracted to purple traps, the colour resembling that of tree bark onto which they oviposit (Lelito et al. 2007; Rodriguez-Saona et al. 2007; Crook et al. 2009; Francese et al. 2010). Finally, females produce a pheromone, (3Z)-lactone, which is attractive to males (Silk et al. 2011; Ryall et al. 2013; Silk and Ryall 2015). We took advantage of these behaviors in our Halifax study by deploying purple and green pheromone-baited traps; this is a common approach in studies of forest pest insects (e.g., Régnière et al. 2013; Parker et al. 2020). Nevertheless, we were surprised by

the ability of EAB to locate mates even in the very low-abundance populations we studied. While EAB are not known to use their pheromones for long-range attraction, we speculate that the multimodal nature of host and mate-finding behaviours in EAB, including attraction to host volatiles, allows them to aggregate on trees and potentially overcome mate-finding Allee effects, at least at the densities measured in our study. In turn, this ability to find mates even at rather low population densities may help explain the rapid spread of EAB (Ward et al. 2020) across its invasive North American range.

Understanding the strength of Allee effects on invasive species remains important, as it can impact how management strategies are best implemented. Invasive species experiencing Allee effects may be better managed at low densities (Tobin et al. 2011), and may have a longer window for detection before new populations are unmanageable. For species like EAB, the lack of a pronounced component Allee effect may explain the difficulties encountered in population management following detection. Eradication may be possible only at extremely early stages of local establishment, and management resources may be better spent reducing human-assisted spread.

Conclusion

Risk modelling of long-distance dispersal of EAB has shown that Allee effects are likely to be an important determinant of its spread (Caouette 2023). However, we have found that EAB does not show a pronounced mate-finding Allee effect even at the lower population densities we were able to study. It is, of course, possible that other component Allee effects are present; further work would be necessary to test such hypotheses. However, the lack of mate-finding Allee effects, in combination with other factors such as the widespread planting of clonal ash cultivars and lack of natural enemies, may contribute to the rapid spread of EAB in North America and elsewhere. Allee effects remain an important and seldom-studied aspect of biological invasions. By studying population dynamics in consideration of Allee effects, we can better understand population growth and invasiveness of non-native species to determine the best methods to manage invasive 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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Author contributions

Conceptualization: Alexandre P. Caouette, Claire E. Rutledge, Stephen B. Heard, Deepa S. Pureswaran. Data Curation: Alexandre P. Caouette. Formal Analysis: Alexandre P. Caouette. Funding Acquisition: Stephen B. Heard, Deepa S. Pureswaran, Claire E. Rutledge. Investigation: Alexandre P. Caouette, Claire E. Rutledge. Methodology: Alexandre P. Caouette, Claire E. Rutledge. Project Administration: Alexandre P. Caouette, Claire E. Rutledge. Resources: Stephen B. Heard, Deepa S. Pureswaran. Software: Alexandre P. Caouette. Supervision: Stephen B. Heard, Deepa S. Pureswaran. Validation: Alexandre P. Caouette. Visualization: Alexandre P. Caouette. Writing – Original Draft Preparation: Alexandre P. Caouette. Writing – Review & Editing: Alexandre P. Caouette, Stephen B. Heard, Deepa S. Heard, Deepa S. Pureswaran, Claire E. Rutledge

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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. We have submmited data to DRYAD at the following URL: https://doi.org/10.5061/dryad.000000097.

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Supplementary material 1

Number of beetles caught in a cluster based on the distance from the invasion epicentre

Authors: Alexandre P. Caouette, Claire E. Rutledge, Stephen B. Heard, Deepa S. Pureswaran Data type: docx

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Research Article

Niche partitioning of invasive Amur sleeper (*Perccottus glenii*) amongst native fish communities in three different freshwater ecosystems

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Abstract

The invasive fish, Amur sleeper, poses a significant and growing threat to Central European freshwater ecosystems. Despite its rapid spread, the ecological implications of its invasion have been poorly explored. Recent findings confirm its presence in various Estonian freshwater systems, raising concerns about its imminent expansion into larger lakes. To better understand its potential ecological impacts, we explored the isotopic niche of the Amur sleeper in comparison with native fish species co-existing in three Estonian freshwater ecosystems. We employed carbon and nitrogen stable isotope analyses alongside gut content analyses. Our findings show that the Amur sleeper's diet in newly-invaded Estonian water bodies predominantly comprises benthic macroinvertebrates, although it may also include fish, confirming its role as a predator in the local food web. Notably, Amur sleeper populations exhibited clear isotopic niche partitioning in three invaded ecosystems. A logistic regression model, based on stomach content analyses, revealed an ontogenetic diet shift from benthivorous to piscivorous feeding habits from small to large specimens. Amur sleeper exhibits voracious, non-selective feeding habits, which can negatively impact native freshwater communities. The ability to occupy a distinct isotopic niche, with minimal overlap with native fish populations, may reduce interspecific competition, facilitating the spread and establishment of Amur sleeper in newly-invaded habitats. Managing the spread of this invasive species thus becomes even more critical to safeguard the integrity of native aquatic ecosystems.

Key words: gut content, invasive species, isotopic niche, native fish community, ontogenetic shift, stable isotopes

Introduction

The spread of invasive alien species is recognised as one of the greatest threats to global biodiversity (Roy et al. 2024). Freshwater ecosystems are particularly vulnerable to biological invasions where invasive species can lead to significant ecological effects, altering community structures and ecosystem processes (Dudgeon et al. 2006; Gallardo et al. 2016). Invasive freshwater fish species can cause strong food web disruption (Wainright et al. 2021), affecting native communities across different trophic levels (Cucherousset and Olden 2011) and exerting critical top-down and bottom-up effects on the food webs of invaded ecosystems (Britton 2023). Amur sleeper (*Perccottus glenii* Dybowski, 1877) is an invasive fish species that has spread from its natural habitat, the River Amur watershed in far eastern Asia (Reshetnikov 2004, 2013; Covaciu-Marcov et al. 2017; Rau et al. 2017) and has invaded many freshwater ecosystems in central and eastern Europe (Nehring and Steinhof 2015; Rechulicz et al. 2015; Reshetnikov and Karyagina 2015; Kutsokon 2017; Nastase et al. 2019; Kutsokon et al. 2021; Pihlström et al. 2022; Djikanović et al. 2023).

Amur sleeper invasion could alter trophic relationships in aquatic communities by generalist feeding on different trophic levels and prey items mainly associated with submerged vegetation (Grabowska et al. 2009). The wide diet spectrum of Amur sleeper and opportunistic predation with a flexible feeding strategy favours its expansion at the expense of native fauna (Grabowska et al. 2009). Amur sleeper can impact ecosystems via competition with and predation on native species (Grabowska et al. 2009; Kati et al. 2015) and by altering habitats (Reshetnikov 2001, 2003; Plyusnina 2008). The recent, rapid expansion of Amur sleeper into central and eastern European waterbodies will threaten the overall health and biodiversity of local aquatic ecosystems (Grabowska et al. 2009; Somogyi et al. 2023). Studies on the diet of wild Amur sleeper populations in Europe have been conducted by analysing gut contents (Koščo et al. 2008), providing a snapshot of its feeding habits and additional studies were conducted in experimental laboratory conditions (Grabowska et al. 2009; Kati et al. 2015; Grabowska et al. 2019). However, more research is needed to better understand the ecosystem effects of invasive Amur sleeper in natural conditions, its potential impacts on native fish communities and how to improve the efficiency of management actions.

Amur sleeper has already been reported in Estonian freshwater ecosystems (Reshetnikov 2010) and invasion of large Estonian lakes, such as Peipsi and Võrtsjärv, is likely. In 2005, a few specimens of Amur sleeper were captured in the outflowing water channel of the Baltic Thermal Power Plant (BPP), while an abundant (approximately 1 individual per m²) population was found in a pond close to the Narva Reservoir (Tambets and Järvekülg 2005; Tambets et al. 2010). A few individuals were also found at another site in the Narva Reservoir close to the pond. Amur sleeper was likely introduced by fishermen into the pond and later, during floodings (the pond becomes connected to the reservoir only during high water levels), some individuals escaped and populated the nearby Narva Reservoir.

In summer 2008, northern pike (*Esox lucius* Linnaeus, 1758) were introduced in the pond to eradicate Amur sleeper. Intensive capture and predation by pike and perch reduced the population biomass of Amur sleeper by 80% (Tambets et al. 2010). Although Amur sleeper biomass decreased sharply, the species remained at a low abundance in the pond during the following years.

Due to its feeding habits, ability to exploit a wide spectrum of food resources and rapid invasion rate, it is crucial to better understand the potential ecological effects of the Amur sleeper on native communities for impact assessment and risk management. In this study, we aimed to: (1) evaluate the diet plasticity of invasive Amur sleeper, (2) assess if the species displays a similar isotopic niche in each of the three different invaded ecosystems and (3) quantify its potential overlap with native fish species. We hypothesised that: 1). Amur sleeper has a wider isotopic niche than benthic native fish and 2). Amur sleeper is a strong competitor for local predatory-benthivorous fish species (e.g. perch) due to niche overlap and a similar diet.

Methods

Sampling area

Samples were collected from the Narva Reservoir (59°21.02'N, 28°10.79'E), the cooling water channel of the Baltic Thermal Power Plant (BPP) (59°18.94'N, 28°5.12'E) and a small pond located near, but not connected, to the reservoir (59°21.19'N, 28°11.17'E) (Fig. 1). Narva reservoir is a large (191 km²) and shallow (average depth 1.8 m) artificial water body located on the Narva River on the border between Estonia and Russia. The reservoir is connected to numerous flowing and stagnant water bodies in the large catchment area of the Narva River (drainage basin 55,800 km²). The reservoir has an extensive favourable habitat for phytophilous and, in some areas, warm-water fish. The photic zone extends to the bottom in large areas, favouring the growth of vegetation. The BPP channel is connected to the reservoir and water in the sampled part of the channel is warmer than in the reservoir depending on BPP operations. The BPP channel is approximately 15 km long, with mean and maximum depth of 1.8 m and 2.5 m, respectively. Surface area of sampled pond is 0.045 km², Secchi depth is 4.5 m and mean and maximum depths are 2.8 m and 7 m, respectively. The bottom of the pond is covered by macrophytes. When we sampled the pond, the oxygen concentration above the macrophyte layer was 8.8 mg/l, while in early spring, this water layer was hypoxic (below 1 mg/l).

Sampling of fish and potential food sources

Samples of Amur sleeper and native fish species were collected in September 2017 from the reservoir, pond and channel using electrofishing and gillnets. Nordic-type, multi-section benthic and pelagic gillnets (5–55 mm from knot to knot) were supplemented with larger mesh sized nets (65 mm from knot to knot). Gillnets were set up overnight in each sampling site and retrieved approximately 15 hours later the next day. Electrofishing (mean area 618 m²) was performed in shallow water



Figure 1. Sampling sites for Amur sleeper, native fish species and macroinvertebrates, as indicated by hollow black circles. Black arrows within the BPP channel indicate the flow direction.

with abundant vegetation near the areas where gillnets were deployed, at the same time or after their removal. The pond was also sampled prior to 2017 and in 2018, using only electrofishing. In September 2017, three replicate samples for macro-invertebrates were collected using a kick-net with a mesh size of 0.5 mm (0.5 to 1.5 m water depth), along littoral areas at sites near where gillnets were set and removed and electrofishing was performed. Additionally, Ekman grab was used to sample macroinvertebrates from the bottom of the pond.

Stable isotope analyses

After sampling, fish and macroinvertebrates were identified to species and at the lowest taxonomic levels, respectively. For stable isotopes analysis (SIA), a piece of white dorsal muscle was collected from each specimen, while macroinvertebrate samples were prepared from whole organisms to represent macroinvertebrate food sources for Amur sleeper (Table 1).

Table 1. Number (n) of macroinvertebrates species sampled in pond, Narva Reservoir and channel and their respective mean values (± standard deviation) of carbon and nitrogen stable isotopes.

Ecosystem	Species	n	d ¹³ C‰	d ¹⁵ N‰
Pond	Gastropoda			
	Bithynia tentaculata	3	-29.58 ± 0.66	3.82 ± 0.46
	Crustacea			
	Asellus aquaticus	5	-25.26 ± 0.63	3.35 ± 0.34
	Ephemeroptera			
	Ephemerellidae	5	-26.92 ± 0.79	2.2 ± 0.27
	Coleoptera			
	Dytiscidae	6	-25.55 ± 2.86	4.81 ± 0.65
	Bivalvia			
	<i>Sphaerium</i> sp	9	-32.22 ± 0.31	3.98 ± 0.36
Narva Reservoir	Gastropoda			
	Radix balthica	4	-27.67 ± 1.00	8.95 ± 0.44
	Crustacea			
	Asellus aquaticus	6	-26.70 ± 0.27	7.73 ± 1.43
	Gmelinoides fasciatus	16	-25.88 ± 0.75	8.43 ± 1.17
	Bivalvia			
	Dreissena polymorpha	4	-30.75 ± 0.23	10.16 ± 0.08
	Odonata			
	Coenagrionidae	3	-29.25 ± 0.12	11.13 ± 0.07
	Trichoptera			
	Phryganeidae	3	-25.40 ± 3.04	10.69 ± 0.47
BPP channel	Gastropoda			
	Lymnaea stagnalis	3	-21.99 ± 0.71	8.18 ± 0.16
	Crustacea			
	Procambarus virginalis	6	-29.09 ± 1.29	8.28 ± 0.62
	Ephemeroptera			
	Caenis horaria	2	-31.02 ± 1.01	6.94 ± 0.12
	Bivalvia			
	Dreissena polymorpha	3	-27.70 ± 0.73	9.3 ± 0.31
	Odonata			
	Aeshnidae	3	-26.66 ± 0.12	9.57 ± 0.02
	Trichoptera			
	Phriganeidae	3	-28.38 ± 0.66	8.84 ± 0.19

All SIA samples were freeze-dried for 48 hours to constant weight, ground to a fine, homogenous powder, weighed into tin cups (~ 0.6 mg of material) and encapsulated. If needed, small macroinvertebrates of the same taxa were pooled to achieve enough material for SIA. Analyses of stable carbon and nitrogen isotopes were conducted using a Thermo Finnigan DELTA_{plus} Advantage continuous-flow isotope ratio mass spectrometer coupled to a Flash EA 1112 elemental analyser (Thermo Scientific, Bremen, Germany) at Jyväskylä University (Finland). SI values for carbon and nitrogen are expressed as parts per thousand (‰) delta values ($\delta^{13}C$, $\delta^{15}N$) relative to international standards:

$$X = (Rsample | Rstandard - 1) \times 1000$$

where X is either carbon or nitrogen SI value and R is the ratio of heavy to light SI of carbon or nitrogen in samples and standards.

Reference materials were used as internal standards, with known relationships to the international standards of The Vienna Pee Dee Belemnite (VPDB) for carbon SI and atmospheric nitrogen (Air- N_2) for nitrogen SI. White muscle tissue of northern pike (*Esox lucius*) and birch leaves (*Betula pendula* L.), with known isotopic compositions, were used as internal working standards to ensure analytical precision. One internal standard was run repeatedly after every five samples in each sequence. Standard deviations within reference samples in each analytical run were always less than 0.1‰ for carbon and 0.2‰ for nitrogen in pike and birch leaf samples. Sample analysis also yielded percentage carbon and nitrogen from which C:N ratios (by weight) were derived.

Isotopic niches

Stable isotope values were used for identifying isotopic niches and to evaluate and compare isotopic niche similarity of Amur sleeper and native fish species in each of the studied freshwater ecosystems. Isotopic niches and isotopic niches overlapping calculations were performed using the *SIBER* (Stable Isotope Bayesian Ellipses in R) package in R (R Core Team 2022). Corrected standard ellipse areas (SEAc) were calculated from the variance and covariance of δ^{13} C and δ^{15} N values, corrected for small sample size, as a measurement of the population niche ellipse area for each fish species (Jackson et al. 2011). Each SEAc was calculated by drawing the core of the isotopic niches (40%) to avoid overestimation due to extreme carbon and nitrogen values of individuals. Isotopic niche overlapping was also calculated to assess the degree of ecological similarity between Amur sleeper and native fish species. The overlap was calculated as the proportion of the sum of the non-overlapping areas between two ellipses and expressed as a percentage, ranging from 0, when the ellipses exhibit no overlap, to 1, when the ellipses overlap completely (Jackson et al. 2019).

Stomach content analysis

Amur sleeper is a predatory, omnivorous fish and its diet was assessed by stomach content analyses to support SIA interpretations. Stomach contents were identified and divided into the following categories: macrophytes, zooplankton, macroinvertebrate parts, gastropods, bivalves, Chironomidae, Trichoptera, Ephemeroptera, *Asellus aquaticus*, Coleoptera, Odonata, fish, Oligochaeta, Gammaridae, Micronecta and detritus. Stomach fullness was estimated, ranging from 0% (empty stomach) to 100% (full stomach), based on the sum of percentages of each food category (Jensen et al. 2012). The percentage of occurrence ($^{\circ}O_{i}$) of food items in Amur sleeper was calculated as:

$$\% O_i = \frac{J_i}{P} \times 100\%$$

where J_i is the number of Amur sleeper individuals containing prey *i* and *P* is the number of Amur sleeper individuals with food in their stomach (Amundsen et al. 1996).

Statistical analyses

To assess the probability of Amur sleeper undergoing an ontogeny diet shift, a logistic regression model was used to estimate the length when individuals could switch their diet from benthivorous to piscivorous feeding habits

$$\gamma = \left[e^{(\alpha+\beta x)}\right] \left[1 + e^{(\alpha+\beta x)}\right]^{-1}$$

where *y* indicates the occurrence of fish in Amur sleeper stomach, *x* is the length of Amur sleeper specimens and α and β are the coefficients estimated by the model.

From stomach content analyses, individuals with fish in their stomachs were given the value of 1, whereas those without fish in their stomach were given the value of 0. The logistic regression model was performed in R (R Core Team 2022) using Generalised Linear Model (GLM) with binomial family, library rms (Regression Model Strategies), with piscivory as the response variable and length as the predictor variable. Residual analyses were conducted for checking deviation from the distribution, residual dependency on predictor and heteroscedasticity, to validate the Generalised Linear Model (GLM). The regression line was plotted (with 95% of confidence interval) between prey fish occurrence in Amur sleeper stomachs (0 or 1) and the length of analysed Amur sleeper specimens. The feeding behaviour shift to piscivory was set at the individual length where the probability of fish occurrence in the stomachs exceeded 50%. According to the individual total length (TL) set by the logistic regression model, all Amur sleeper specimens were divided in small (TL < 8.7 cm) and large (TL > 8.7 cm) groups. Differences in carbon and nitrogen stable isotopes between small and large Amur sleeper individuals, in each studied ecosystem, were tested using the Mann-Whitney U test. Assumptions for normality and homogeneity of variances were tested prior statistical analyses using Shapiro-Wilk and Levene tests. Variables were transformed if assumptions were not met. All statistical analyses were performed in R (R Core Team 2022).

Results

A total of 66 Amur sleeper individuals were caught: 17 from the pond (4 large and 13 small), 36 from the Narva Reservoir (8 large and 28 small) and 13 from the BPP channel (1 large and 12 small) (Table 2). Amongst the three populations, the smallest individuals appeared in the BPP channel (mean length = 4.50 ± 2.0 cm) compared to the reservoir (7.01 ± 3.27 cm) and pond (7.55 ± 4.45 cm; Table 2). All Amur sleeper individuals collected in the three ecosystems were caught by

		1		,	1	1	,
Ecosystem	Fish species	n	Length (cm)	d ¹³ C‰	d ¹⁵ N‰	SEAc ³	SEAc overlapping%
Pond	Amur sleeper (<i>Perccottus glenii</i>) large	4	14 ± 5.2	-38.19 ± 0.59	9.58 ± 1.82	77.5	-
	Amur sleeper (Perccottus glenii) small	13	5.6 ± 1.0	-38.16 ± 10.61	6.79 ± 2.48	_	_
	Roach (Rutilus rutilus)	10	20.0 ± 5.1	-38.17 ± 0.95	7.88 ± 0.23	0.43	0
	Rudd (Scardinius erythrophthalmus)	5	10.9 ± 2.8	-26.47 ± 1.12	8.10 ± 0.10	0.49	0
	Perch (Perca fluviatilis)	9	15.9 ± 2.9	-26.06 ± 1.12	8.80 ± 0.37	1.51	0
	Pike (Esox lucius)	4	17.2 ± 3.0	-26.33 ± 0.14	8.08 ± 0.46	0.27	0
Narva	Amur sleeper (<i>Perccottus glenii</i>) large	8	11.5 ± 2.8	-27.04 ± 0.28	12.86 ± 0.27	0.88	-
Reservoir	Amur sleeper (Perccottus glenii) small	28	5.7 ± 2.0	-27.13 ± 0.52	11.83 ± 0.38	_	_
-	Roach (Rutilus rutilus)	11	20.8 ± 2.0	-26.46 ± 1.68	13.27 ± 0.75	4.81	14
	Rudd (Scardinius erythrophthalmus)	8	22.1 ± 2.6	-24.10 ± 1.86	11.69 ± 1.53	10.7	0
	Perch (Perca fluviatilis)	12	23.4 ± 3.4	-26.62 ± 0.66	14.89 ± 0.67	1.53	0
	Ruffe (Gymnocephalus cernua)	8	6.8 ± 2.5	-30.02 ± 2.36	11.78 ± 1.45	5.19	0
	Tench (Tinca tinca)	4	31.3 ± 8.7	-27.19 ± 0.81	12.87 ± 0.62	2.09	35
	Bleak (Alburnus alburnus)	5	11.3 ± 0.8	-29.31 ± 1.28	12.61 ± 0.50	1.66	0
	Spined loach (Cobitis taenia)	5	9.4 ± 0.3	-27.78 ± 0.42	12.37 ± 0.28	0.50	32
	White bream (Blicca bjoerkna)	6	12.6 ± 1.6	-26.08 ± 0.64	14.01 ± 0.57	1.71	0
BPP channel	Amur sleeper (<i>Perccottus glenii</i>) large	1	9.2	-27.64	11.35	0.59	_
-	Amur sleeper (Perccottus glenii) small	12	4.1 ± 1.6	-29.0 ± 0.45	10.52 ± 0.48	_	-
	Roach (Rutilus rutilus)	5	12.1 ± 1.4	-27.04 ± 1.13	12.65 ± 0.91	4.95	0
	Rudd (Scardinius erythrophthalmus)	5	23.1 ± 3.7	-23.61 ± 1.33	11.74 ± 0.84	5.93	0
	Perch (Perca fluviatilis)	6	19.3 ± 3.5	-26.46 ± 1.39	13.28 ± 0.17	0.93	0
	Spined loach (Cobitis taenia)	5	7.4 ± 1.1	-28.74 ± 0.71	12.24 ± 0.54	1.46	0

Table 2. Number of Amur sleeper and other fish species individuals used for stable isotope analyses and mean values of their length (cm) and carbon and nitrogen stable isotope values (± standard deviation). SEAc indicates the Standard Ellipse Area corrected of each fish population isotopic niche in the pond, Narva Reservoir, and BPP channel. SEAc of Amur sleeper is calculated including large and small specimens. SEAc overlapping % represents the degree of isotopic niche similarity between Amur sleeper and other fish species in each ecosystem.

electrofishing, CPUE was highest in the channel, followed by the reservoir and pond (Table 3). Perch (*Perca fluviatilis*), roach (*Rutilus rutilus*), rudd (*Scardinius erythrophthalmus*) and white bream (*Blicca bjoerkna*) had highest CPUE amongst the fish species collected with gillnets (Table 3).

Isotopic niche widths and overlap of Amur sleeper and native fish communities

SIA results show that values of nitrogen stable isotope of Amur sleeper individuals differ between the pond and reservoir and between the pond and channel, being highest in the reservoir and lowest in the pond (Fig. 2). However, the nitrogen isotope mean values of large Amur sleeper individuals were slightly higher than those of small specimens in the pond (p-value = 0.07) and in the reservoir (p-value < 0.001), while carbon isotope mean values were similar in the pond (p-value = 0.99) and reservoir (p-values = 0.53) (Fig. 2). However, in the pond, small Amur sleeper specimens showed substantial variation of carbon isotope values, with some individuals displaying exceptionally low numbers (Figs 2, 3). Carbon and nitrogen isotopic differences between large and small Amur sleeper individuals in the channel were not tested since, according to the logistic regression model, only

Place and sampling type	Amur sleeper	Perch	Pike	Roach	Gibel carp	Rudd	Ruffe	Spined loach	Bleak	Tench	White bream
Pond											
Electrofishing	0.2	0	0.3	0	0	0	0	0	0	0	0
Benthic Nordic	-	18.3	0.33	4.3	0	3	0	0	0	0	0
Pelagic Nordic	-	11	0	6	0	6	0	0	0	0	0
Benthic 65 mm (knot to knot)	-	0	0	0	0	0	0	0	0	0	0
Narva Reservoir											
Electrofishing	6	0	0.1	0	0	0	0	0.3	0	0.5	0
Benthic Nordic	-	10.3	0	6.3	0	0.7	6.3	0.7	0	0.3	0.3
Pelagic Nordic	-	0	0	20	0	1	0	0	7	0	0
Benthic 65 mm (knot to knot)	-	0	0	0	0	0	0	0	0	2	0
BPP channel											
Electrofishing	43	0	0.1	0	0	0	0	13	0.4	0	0
Benthic Nordic	-	10.3	0	11.3	0	10.7	0	0.3	0.3	0	1
Benthic 65 mm (knot to knot)	_	0	0	0	4	0	0	0	0	0	0

Table 3. Catch Per Unit Effort (CPUE) indicates the number of fish per 100 m² of sampling area in electrofishing and the number of fish per net in gillnet sampling, caught in pond, Narva Reservoir and BPP channel.

one individual belonged to the large group. The nitrogen isotopic values of Amur sleeper were between native fish and macroinvertebrate communities at all sampling sites, although large specimens were located at the same level with native fish species in the pond (Fig. 2).

SIBER model results indicated that Amur sleeper isotopic niches did not overlap with those of co-occurring native fish populations, except in Narva Reservoir, where it overlapped with tench (35%), spined loach (32%) and roach (14%). The isotopic niche (SEAc) of Amur sleeper was very wide in the pond (77.5‰²) due to low carbon isotope values, while the niches were smaller in the channel and Reservoir (0.59‰² and 0.88‰², respectively), compared to other fish species within each system. Only spined loach in the reservoir occupied a smaller niche area compared to Amur sleeper (Fig. 3, Table 2).

Stomach contents

The logistic regression curve indicated that Amur sleeper undergoes an ontogenetic shift from < 50% benthivorous to > 50% piscivorous diet at an individual total length (TL) of 8.7 cm (p = 0.004; 95% CI, Fig. 4).

Stomachs of all sampled Amur sleeper individuals (66) were analysed, of which 65 were 100% full, regardless of fish size and one was 90% full. The amount of the analysed stomachs of large and small individuals were respectively 4 and 13 from pond; 8 and 28 from Narva Reservoir and 1 and 12 from the channel. In all the invaded ecosystems, the Amur sleeper diet comprised mainly macroinvertebrates and, to some extent, fish. In the channel, only one individual represented the large group (TL > 8.7 cm), its stomach being 100% full of macrophytes and detritus. In all studied ecosystems, the diet of small (TL < 8.7 cm) specimens consisted mostly of macroinvertebrates (fragments), ranging from 17% in the channel to 46% in the pond, with Chironomidae, Trichoptera, Ephemeroptera and Odonata being the most common (Fig. 5). Larger (TL > 8.7 cm) Amur sleeper individuals had





mainly fish in their diets, with the highest proportions in the reservoir (88%) and pond (50%) (Fig. 5). However, fish were also found in some smaller specimens, accounting for 4% of stomach contents in the reservoir and 17% in the channel. Zooplankton (14%) and Gammaridae (7%) were found only in stomachs of



Figure 3. Isotopic niches of each fish species represented by ellipses and fish individuals represented by open circles in the (**A**) pond, (**B**) Narva Reservoir and (**C**) BPP channel.

smaller individuals in the reservoir. Although large prey items for Amur sleeper, gastropods and bivalves were also found in stomachs of both size-classes, with relatively high occurrence of gastropods in larger individuals in the pond and reservoir and with lesser occurrences of gastropods and bivalves in smaller specimens in the reservoir and channel (Fig. 5).



Figure 4. Logistic regression curve was fitted by the following equation $y = [e^{(-13-226 + 0.426x)}][1 + e^{(-13-226 + 0.426x)}]^{-1}$ showing ontogenetic diet shift from benthivory (0) to piscivory (1). Shadow area represents 95% Confidence Interval.



Figure 5. Prey items occurrence (%) in stomach contents of (**A**) large (total length > 8.7 cm) and (**B**) small (total length < 8.7 cm) Amur sleeper individuals in the pond, Narva Reservoir and BPP channel.

Discussion

Invasive Amur sleeper was well-established in each of the studied waterbodies and occupied the isotopic niche between predatory-omnivorous fish species and macroinvertebrates. In the reservoir and channel, the nitrogen isotope values of Amur sleeper, both large and small, show its intermediate role between bottom-up and top-down energy fluxes of the food web, while in the pond, large individuals had similar isotope values with native fish species. Yet, in the pond, the isotopic niche of Amur sleeper was much wider than those of co-occurring native fish. Substantial variations in nitrogen and carbon isotope values and much lower values for Amur sleeper compared to other fish, contributed to the wide isotopic niche in the pond. Low carbon SI values may indicate anoxic conditions at the pond bottom, favouring methanogenesis and conversion of methane to microbial biomass by methane-oxidising bacteria (MOB) (Jones and Grey 2011). MOB that have low carbon SI values can be consumed by invertebrate consumers, which transfer low SI value up to the top predators (fish) through trophic transfer (Ravinet et al. 2010; Sanseverino et al. 2012). Low carbon SI values and the wider isotopic niche of Amur sleeper in the pond may thus be the result of its occasional feeding on profundal macroinvertebrates that have consumed MOB (Eller et al. 2005; Jones and Grey 2011; Grey 2016). Exceptionally low carbon isotope values of small Amur sleeper individuals in pond indicate their higher assimilation of methane-derived carbon if compared to large individuals. Such feeding behaviour of small individuals widen the overall isotopic niche of Amur sleeper in the pond, with some individuals using energy sources mostly from profundal and some from littoral habitats.

Chironomidae and Oligochaeta feeding on MOB would be the best prey candidates for low carbon SI values. Unfortunately, we were not able to analyse SI values from these organisms, since the bottom of the pond was fully covered by macrophytes, preventing sampling of profundal benthic macroinvertebrates. However, amongst the sites and both size groups, highest proportions of Oligochaeta were found from stomachs of small Amur sleeper in the pond, suggesting that Oligochaeta was an important prey item and potential source for methane-derived carbon.

Invasive Amur sleeper is a predatory-omnivorous fish species mainly feeding on macroinvertebrates, but can be also piscivorous (Grabowska et al. 2009; Rau et al. 2017; Djikanovic et al. 2023). We hypothesised that its isotopic niche would consistently overlap with other fish species, particularly predatory-benthivores inhabiting littoral habitats, such as roach and perch. However, the isotopic niche of Amur sleeper only marginally overlapped with some native fish species (tench, spined loach and roach) in Narva Reservoir, but did not overlap with native fish in the pond or BPP channel. Results also indicated isotopic niche partitioning between Amur sleeper and co-occurring native fish populations. The ability of Amur sleeper to occupy an isotopic niche with limited overlap with ecologically similar native fish species may suggest an adaptation strategy, aimed at avoiding interspecific competition during its invasion and establishment in new ecosystems. Furthermore, we hypothesised that Amur sleeper has a wider isotopic niche than benthic native fish; however, our results showed the opposite. The Amur sleeper exhibited a narrower isotopic niche, indicating the use of different habitats and food sources compared to co-occurring native fish. These observations underscore the high level of adaptability and plasticity of Amur sleeper. However, the isotopic niche area of Amur sleeper could also depend on the diversity of native fish

community. A higher diversity of native fish community could imply higher interspecific competition, potentially leading to a reduced isotopic niche area of the invasive species. Conversely, lower diversity might allow for an expanded isotopic niche area for the invasive species (López-Rasgado et al. 2016). The relationship between niche width and community diversity is an intriguing aspect of Amur sleeper invasion and future isotope studies should focus on analysing more invaded ecosystems across a larger spatial scale.

In accordance with prior research (Koščo et al. 2008; Grabowska et al. 2009), our findings showed that Amur sleeper undergoes an ontogenetic dietary shift from benthivory to piscivory at a total length of 8.7 cm (± 95% CI), which is 1.7 cm longer than the shift size reported previously (Koščo et al. 2008; Kutsokon et al. 2021). The dietary differences between small and large specimens show a shift from smaller, more readily available prey to larger, more nutritious prey during the growth. This transition reflects changes in their ecological role, feeding capabilities and impact on the ecosystem. Our findings align with the results of previous studies (Koščo et al. 2008; Grabowska et al. 2009; Kati et al. 2015; Kutsokon et al. 2021), which observed that small Amur sleepers primarily consume high proportions of small and less motile macroinvertebrates, such as Chironomidae, Trichoptera, Ephemeroptera and Odonata. In contrast, larger Amur sleepers show a higher consumption of larger macroinvertebrates like gastropods and bivalves, as well as of more motile prey, such as Coleoptera and fish. In the pond and reservoir, where the Amur sleeper populations exhibited a greater occurrence of larger individuals, the proportions of fish prey found in their stomachs were higher compared to their smaller counterparts in the channel. A previous study conducted in the same pond found that Amur sleeper's diet consisted mainly of juveniles of its own and sunbleak (Leucaspius delineatus) (Tambets et al. 2010), showing high cannibalistic behaviour, as also reported in another study (Grabowska et al. 2009). Moreover, Tambets et al. (2010) found that the fish community in the studied pond was represented mostly by adult Amur sleeper, indicating strong predation on smaller individuals, not only by perch and pike, but also by larger conspecifics.

Pike were introduced in the pond to help control the Amur sleeper population. Similar to the findings of Rakauskas et al. (2019), pike specimens had a high growth rate and their food consisted exclusively of Amur sleeper individuals. This top predator could limit opportunities for Amur sleeper to prey on macroinvertebrates and forage in littoral habitats, thus increasing its reliance on piscivory and cannibalism. However, Amur sleeper individuals in our study had stomachs full of consumed food items, regardless of body size, highlighting its voracious feeding habits (e.g. Djikanovic et al. (2023)).

Amongst the prey items found in Amur sleeper stomachs, spined loach (*Cobitis taenia*) was identified. Spined loach is a protected fish species in Estonia (Kesler et al. 2009; Tammiksaar and Kangur 2020). The isotopic niches of Amur sleeper and spined loach overlapped substantially in the reservoir. This overlap, along with direct predation, suggests that the Amur sleeper may pose a significant threat to the endangered spined loach population in invaded habitats. These findings also suggest that Amur sleeper can threaten the protected weatherfish (*Misgurnus fossilis*), which has similar habitat preferences (Djikanović et al. 2023) and inhabits areas upstream of the Narva Reservoir, to where the Amur sleeper has not yet spread.

Interestingly, amongst species consumed by Amur sleeper, there were also different invasive species present, such as zebra mussels (*Dreissena polymorpha*),

amphipod (*Gmelinoides fasciatus*) and marbled crayfish (*Procambarus virginalis*), which are all well established in the studied waterbodies. All these invasive species occupy different trophic positions and could represent food sources for Amur sleeper, possibly facilitating Amur sleeper invasion and establishment. These results support the invasion meltdown hypothesis (Simberloff and Von Holle 1999), where the establishment of one invasive species can facilitate the establishment of others.

In our studied ecosystems, Amur sleeper can affect native communities at different trophic levels, especially native macroinvertebrate communities. These feeding characteristics exert both top-down and bottom-up effects, disrupting energy transfer to higher trophic levels. In a recent study, Kuparinen et al. (2023) quantified the potential effects of Amur sleeper on the food web of the not-yet-invaded, large Estonian Lake Võrtsjärv. Results showed that Amur sleeper invasion would decrease the biomass of native top predators, such as pikeperch, perch, pike and eel, which all have high fishery value, due to competition for benthic macroinvertebrate food and direct predation of the young native fish individuals. In contrast, the biomass of fish species inhabiting lower trophic levels, such as bream and smelt, was projected to increase, likely as consequence of reducing predation from the top predator.

Our findings showed that Amur sleeper may affect native fish communities in the studied ecosystems, serving as a warning for stakeholders and resource managers of the potential threats that this invasive fish species can pose on invaded and nearby, connected aquatic habitats.

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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

FE and MK- Conceptualisation. MT, EK and FE - Investigation. FE, MK, PT and AT- Formal analysis, data curation, methodology, software and visualisation. FE, MK, PT and TN - Writing original draft. FE, MK, PT, EK, MT, AT and TN - Writing, review and editing.

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Data availability

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

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Research Article

Interactions between invasive pests and pathogens in a native chestnut forest

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Abstract

The introduction in the same area of different invasive species can result in novel interactions, with unpredictable consequences. We carried out a study in Galicia (northwestern Iberian Peninsula) with the aim of clarifying the interactions between two invasive species Cryphonectria parasitica and Dryocosmus kuriphilus. In 2018, we selected five chestnut plots affected by both species. We compared trees affected only by the insect and trees affected by both the insect and the fungus with respect to attack level, gall characteristics, female size and fecundity, and concentrations of nitrogen, water and secondary metabolites. We also evaluated female preferences in a greenhouse assay. There were higher levels of attack in trees affected by both invaders. However, the greenhouse assay showed that ovipositing females do not preferentially choose trees attacked by the fungus. The presence of the fungus had no effect on the size, wall thickness, or hardness of D. kuriphilus galls, but larvae were smaller in trees also affected by the fungus. The fecundity of females was strongly related to the presence of chestnut blight; the number of eggs per female was almost double in trees affected by the fungus. There were no relations between blight and the nitrogen or water content in the galls, where the insects feed. There were also no effects of chestnut blight on the concentration of terpenes or phenols, but condensed tannins were higher in trees with chestnut blight. The higher tannins induced by chestnut blight may directly or indirectly benefit gall wasps. Positive relationships between condensed tannin concentration and reproductive performance of other gall makers were previously reported. Tannins can also improve the negative effects of environmental conditions inside the gall. Our results indicate that the presence of chestnut blight can increase the suitability of chestnut trees for the invasive insect, D. kuriphilus, through the increase in tannins due to the presence of the fungus.

Key words: Asian chestnut gall wasp, Castanea sativa, chestnut blight, interactions, invasive species

Introduction

The number of invasive pathogens and insect pests in forest ecosystems has increased dramatically in the last century, mainly due to the growth of international trade and the associated increase in the movement of plants, wood and wood products (Roques et al. 2009; Walther et al. 2009; Santini et al. 2013; Freer-Smith and Webber 2017). Once established in the new environment, nonnative pests



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Copyright: [©] María Flora Romay-Río et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). can have negative economic and ecological consequences for forest ecosystems. Nonnative pests can cause growth losses and tree mortality (Aukema et al. 2011; Bonello et al. 2020), act as vectors or facilitate the entry in the plant of other damaging organisms (Meyer et al. 2015) and interfere with ecosystem services by reducing biodiversity and wildlife habitats, or by altering natural landscapes and diminishing their cultural value (Liebhold et al. 1995; Boyd et al. 2013). Also, the invasive forest pests and diseases may affect the ability of forests to sequester carbon, protect watersheds or combat desertification (Boyd et al. 2013; Seidl et al. 2018). In the most dramatic cases, these invasives can lead to functional extinction of the afflicted tree species (e.g., chestnut blight, Dutch elm disease, and emerald ash borer). The introduction in the same area of different invasive species can result in novel interactions, with unpredictable consequences such as the emergence of new associations among plant enemies (Santini and Battisti 2019) and new hybrid enemies (Brasier 2001; Brasier et al. 2004).

The European chestnut, *Castanea sativa* Mill., is widely distributed in Europe and Western Asia in natural and semi-natural forests, as well as in plantations and has been cultivated to produce fruit and wood since ancient times (Conedera et al. 2004). In Spain, chestnut occurs mainly in the north, occupying an area of more than 100,000 ha, of which some 45,000 ha are in Galicia (MARM 2011) where this study was conducted. Global chestnut production has increased continuously over the last 40 years (Freitas et al. 2021) and Spain is among the top three chestnut producers in the world (Freitas et al. 2021; Fernandes et al. 2022).

Chestnut blight (Cryphonectria parasitica (Murr.) Barr.) originally from Asia, has been present in southern Europe since the mid-20th century, probably introduced from North America (Dutech et al. 2012). It was detected in Italy in 1938 (Biraghi 1946) and in Spain in 1947 (Elorrieta 1949). Symptoms of chestnut blight involve swelling and cracking of the bark at the infection point on the trunk and branches, which often becomes populated with yellow-orange fruiting bodies. The fungus grows in the inner bark (phloem) and cambium tissue and progresses rapidly from initial infection to large sunken or swollen stem cankers that lead to complete girdling of the twigs, branches or the stems causing them to wilt (Prospero and Rigling 2016; Kolp et al. 2018). However, not all cankers girdle infected stems, canker expansion depends on tree resistance (Hebard et al. 1984), pathogen virulence (Griffin et al. 1983; Enebak et al. 1994), and other abiotic and biotic factors such as associated fungi (Kolp et al. 2018; Kolp et al. 2020). Susceptibility to this pathogen varies widely among *Castanea* species (Graves 1950; Dane et al. 2003; Mellano et al. 2012). Asian species are tolerant to C. parasitica, possibly because of a shared evolutionary history, but the European and North American chestnut species are highly susceptible to the pathogen, and infection normally results in tree mortality (reviewed in Griffin et al. 1983; Lovat and Donnelly 2019). The fungus functionally eliminated chestnut trees from the forest canopy in North America, and American chestnut exists today primarily as an under-story shrub emanating from preexisting root systems (Paillet, 2002). In the European chestnut, the fungus might persist for years in the same tree before the tree dies.

Dryocosmus kuriphilus Yasumatsu (Hymenoptera, Cynipidae) is a gall maker wasp, considered one of the most important chestnut pests worldwide (EPPO 2005). The insect, native to China, spread to other parts of the world includ-

ing Europe, where it was detected in 2002 in northwestern Italy (Brussino et al. 2002). From there it expanded rapidly throughout Europe, reaching Spain in 2012 (DOGC 2012; Pujade-Villar et al. 2013). The formation of the galls interferes with the normal development of the vegetative and reproductive structures of the chestnut tree, reducing wood and fruit production (Kato and Hijii 1997; Battisti et al. 2014; Sartor et al. 2015; Marcolin et al. 2021), al-though damage may vary depending on the forest structure (Castedo-Dorado et al. 2023a).

Galling insects modify plant physiology by inducing the development of complex gall structures in the plant tissue (Harper et al. 2004). Host chemistry can be important both positively and negatively for larval performance of galling insects (Rehill and Schultz 2012; Kot et al. 2018). Numerous reports show that galling insects can manipulate plant defenses to their own advantage (Schultz 1988; Hartley 1998; Oliveira et al. 2016; Lombardero et al. 2022). Increases in terpenes due to gall makers attack might have direct toxic effects on the attacking insect (Naidoo et al. 2018) or attract parasitoids (Borges 2018). Increases in terpenes were reported after D. kuriphilus attack in C. mollissima (Wang et al. 2024). Phenolics include many compounds with toxic or repellent effects towards insects and microorganisms (Lindroth and Hwang 1996). Nevertheless, the role of phenols for gall makers is ambiguous. Galls may contain lower (Nyman and Julkunen-Tiitto 2000; Allison and Schultz 2005) or higher (Hartley 1998) phenolic concentration compared with ungalled tissue. Some phenols are expressed as tannins, which are antibiotic and antixenotic, and have long been associated with pathogen and herbivore defense (Feeny and Bostock 1968; Griffin and Elkins 1986). Tannins can be dramatically elevated in galled plant tissue (Cornell 1983) including galls of our study system (Lombardero et al. 2022). High tannin concentrations in galls have been interpreted as a protection to the larvae against damaging agents (Cornell 1983; Taper et al. 1986; Schultz 1992).

Terpenes might also function in defense against fungal pathogens in conifers (Zeneli et al. 2006). Changes in phenolic composition in response to injury or fungal infection have been considered an active defense response (Brignolas et al. 1995; Evensen et al. 2000; Viiri et al. 2001), including in *Castanea sativa* (Dinis et al. 2011; Camisón et al. 2019). Tannin deposition has been interpreted as a localized host defense against advancing mycelia during active chestnut blight infection (Lovat and Donnelly 2019). Tannin profile differences between Asian *Castanea* species and *C. dentata* and *C. sativa* may explain some differences in blight tolerance between these two species groups (Elkins et al. 1979; Griffin and Elkins 1986; Cooper and Rieske 2008). However, Cook and Wilson (1915) suggested that tannins were used by *C. parasitica* as a source of nutrition.

Both invasive species, *D. kuriphilus* and *C. parasitica*, can colonize the same trees. Since the entrance of *D. kuriphilus* into Europe, numerous associations have been described between this species and native or introduced pathogenic fungi (Seddaiu et al. 2017; Morales-Rodríguez al. 2019), including reports of higher incidence of *C. parasitica* (Meyer et al. 2015; Vannini et al. 2018). We conducted a study in Galicia (northwestern Iberian Peninsula), one of the most important areas for chestnut production in southern Europe, with the aim of testing for interactions between the two invasive species since it could have implications for chestnut management.

Materials and methods

We conducted studies in the field and in greenhouses to assess potential interactions between *D. kuriphilus* and *C. parasitica* living on the European chestnut.

Field sampling

From January to March of 2017, we established 16 study plots to follow the damage caused by *D. kuriphilus* on *C. sativa* in Galicia (see more details in Lombardero et al. 2021). Five of the plots studied by Lombardero et al. (2021) also had trees attacked by *C. parasitica*; these plots were used for the present study. The number of trees per plot that were affected by both the pathogen and the gall wasp ranged from 5 to 9 and the number of study trees attacked only by *D. kuriphilus* ranged from 12 to 18. The plots were in the inner part of Galicia (Fig. 1) and share similar climatic conditions (mean annual temperature 10–12 °C, total annual precipitation 1100–1350 mm; more details in Castedo-Dorado et al. 2023a). The distance from one plot to the next nearest plot ranged from 1.2 to 19 km. The plots were in private chestnut plantations intended for wood production or both wood and nut production. Trees were 21–22 years old and spaced evenly at 4×4 m to 8×8 m.

Greenhouse assay

In 2018, we used a greenhouse assay to test whether D. kuriphilus female adults tend to avoid or prefer plants affected previously by C. parasitica. Study plants were obtained from a nursery with a known outbreak of C. parasitica in their seedlings. The infection occurred in the same year of the study (due to contamination during the common practice of grafting seedlings onto rootstocks resistant to Phytophthora cinnamomic). We selected 54 seedlings (2 years old) of similar size, half of which were infected by the fungus and half were not infected. We ensured that the uninfected plants had no fresh wounds or growth cracks that might have made them susceptible to cross-infection. The 54 plants were divided in three treatments: 10 plants with no chestnut blight and no exposure to D. kuriphilus (Control); 17 healthy plants exposed only to attack of D. kuriphilus (DK); and 26 plants infected with chestnut blight and exposed to D. kuriphilus attack (DK + CP). One of the fungal-infected plants was harvested to isolate and confirm the fungal identification. Plants were kept in 38-liter pots with similar commercial substrate and irrigation system in the experimental greenhouse of the University of Santiago de Compostela on the Campus of Lugo. Control plants remained inside the greenhouse (temperature night-day 18-24 °C and 80% moisture) during the wasp flight season and covered with anti-thrips mesh. The two groups of experimental plants were moved to an open area next to the greenhouse where they were exposed to wild populations of D. kuriphilus that had been well-established in the area since at least 2012 (Pérez-Otero and Mansilla 2012). Trees that were- and were not infected with chestnut blight were interspersed in a grid of approximately 1.5×1.5 m. In 2018, all the plants (except the controls) were outside during the flight season of D. kuriphilus (from late June to middle August) to test if the ovipositing insects preferred or avoided trees infected by C. parasitica. In 2019 and 2020, we repeated the study with the same plants (except 8 affected by chestnut blight that died in the second year) but including controls, outside, to also test if the insect preferred plants that had been attacked the previous year or non- attacked plants (the controls from previous year).



Figure 1. Location of the study plots. Plots are in Central Galicia, in the northwestern Iberian Peninsula.

Attack level

In summer 2018, we selected two branches at random in 17–27 study trees in each of the five field study plots (12–18 trees per plot without *C. parasitica* and 5–9 trees with *C. parasitica* (total of 81 and 36 trees without and with *C. parasitica*, respectively). We did our best to match infected and uninfected trees with respect to size and location in the plot. The different number of trees selected was due to the different availability of trees among plots.

In each branch, we located and examined the portion of the shoot that grew in the previous summer (2017). Within that length of shoot, we counted the number of buds that were present at the end of previous year's growing season (2017) as well as the galls produced in the current year (2018) from these buds. The resulting data allowed us to estimate galls per shoot (adjusted for number of buds per shoot) for each tree. We did not use the shoot of the current year because it was still growing after the insect flight ceased, and it is possible that new buds appeared that were not exposed to the attack. In the greenhouse study, we assessed attack level by counting total galls per tree in the study plants growing in pots.

Larval weight and adult fecundity

In June of 2018, to test if the presence of the pathogenic fungus influenced the growth of *D. kuriphilus* larvae, we measured the dry mass of individual late-instar larvae feeding on trees attacked only by the insect and on trees attacked by both invasive species, respectively. We were able to measure 3–9 larvae from each of 48 trees (8–10 trees per plot, half with and without *C. parasitica*).

We assessed female fecundity by counting the number of eggs produced by 1-10 emerging female adults captured from each of 43 trees (5–12 trees per study plot, 16 with *C. parasitica* and 27 without). We also counted the number of eggs from 10 females that emerged from greenhouse plants affected by chestnut blight and 10 from plants attacked only by *D. kuriphilus*.

Gall characteristics

In June 2018, at each of the five field study plots, we collected and measured 1–4 leaf galls from each of 5–11 trees (total of 76 trees; 34 with the fungus and 42 without). In the laboratory, we measured three perpendicular axes of each gall with digital calipers and averaged them to estimate gall diameter. We also measured the toughness of galls with a penetrometer of small fruits (FT02; Oremor). All galls were subsequently dissected to measure gall wall thickness (with a caliper) and count the number of feeding chambers (each representing one gall wasp progeny).

Effect of the presence of chestnut blight on tree nutritional quality and palatability

In summer of 2018, from each of five field study plots, we collected 2 leaves (one galled and other ungalled) from 6–18 trees (total of 69 trees, 34 with *C. parasitica* and 35 without). In the lab, we then measured total phenols, condensed tannins, and terpenes separately in ungalled leaves (ungalled leaf), in the gall itself (gall), and in the leaf tissue surrounding the gall (galled leaf). In the same sampling, we also collected another five trees per treatment and per plot to analyze water and nitrogen content. Again, we analyzed separately the gall itself and the leaf tissue surrounding the gall and control leaves (ungalled).

Similar measurements were carried out in the greenhouse plants. We sampled one leaf from five control trees (control), and two leaves (one galled and one ungalled) from 20 trees, 10 trees affected only by *D. kuriphilus* alone, and 10 trees affected by both insect and fungus. We also analyzed nitrogen and water content from five leaves from control trees, five from trees attacked by *D. kuriphilus* and five from trees attacked by both insects and fungi. For galled leaves, we analyzed separately gall tissue and the leaf tissue surrounding the gall.

We analyzed concentrations of total terpenes following Wainhouse et al. (1998). Terpene compounds were quantitatively extracted twice with n-hexane (with each extraction including 25 minutes in an ultrasonic bath) from 1 g of leaf or gall from each sample after cutting it into very small sections. Then, the plant material was recovered by filtration, the solvent was evaporated, and the mass of the non-vola-tile terpene residue was measured with a precision scale.

Phenolics were extracted from 0.5 g of plant tissue with aqueous methanol (1:1 vol:vol) in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract (Sampedro et al. 2011). Total phenolic content was determined colorimetrically using Folin-Ciocalteu (Pérez et al. 2023) in a BioTek Elx 850 microplate reader at 740 nm quantified with a standard curve of tannic acid and expressed as mg tannic acid equivalent per g dry mass of plant tissue.

We analyzed condensed tannins following the protocol of Waterman and Mole (1994). The same extract used for phenolic analysis was assayed with butanol – hydrochloric acid reagent (0.7 g ferrous sulphate heptahydrate in 50 mL concentrated HCl and n-butanol added to make 1 L), and absorbance was measured at 550 nm with the same microplate reader, using as standard purified condensed tannins of quebracho (*Schinopsis balansae* Engl., Unitan Saica, Buenos Aires).

To analyze water and N content, samples were weighed fresh and then oven-dried at 60 °C for 48 hours. The dried samples were milled to a fine powder and submitted to instant oxidation (as 0.1 g tissue samples); the gases released were identified with a conductimeter. Analyses were performed by the analytical unit of the University of Santiago de Compostela (RIAIDT).

Statistical analysis

Statistical analyses of the field plots followed earlier studies of this system (Lombardero et al. 2021, 2022). Trees were regarded as the experimental units (Neuvonen and Haukioja 1985). The five study plots, which each contained replicate study trees with and without *C. parasitica*, were treated as a fixed effect because we were specifically interested in these plots with known histories (i.e., our study plots were not a random sample from a large population). Population abundance, measured as galls per shoot, was analyzed with a general linear model (Gaussian distribution) that included plot, presence of blight, and their interaction as fixed effects, tree within plot as a random effect, the number of buds per shoot (potential sites for galls) as a continuous variable (covariate), and no intercept (Lombardero et al. 2021). We did not include an intercept in our models because, based on biological knowledge, we know that when there are zero buds, there are also zero galls.

For the remaining variables, where there were multiple measurements per tree, we calculated an average for each tree and used the tree averages for statistical analyses (Neuvonen and Haukioja 1985); this avoided the hazards of pseudoreplication (Hurlbert 1984) and the complication of unequal sample sizes in nested models. Larval size, adult fecundity, size of galls, toughness, wall thickness and number of cells per gall were analyzed with an ANOVA that included plot, the presence of blight, and their interaction as fixed effects. Prior to analyses, to improve normality and homoscedasticity, larval cells per gall were log-transformed.

Water and nitrogen content, terpenes, phenols and condensed tannins, were analyzed with an ANOVA that included plot, the presence of blight, the type of leaf tissue analyzed (ungalled leaf, ungalled portion of galled leaf, or gall), and their interactions as fixed effects and tree nested with plot and the presence of *C. parasitica* as random effect. Prior to analyses, to improve normality and homoscedasticity, terpenes, phenols, and condensed tannins were square root-transformed. Each replicate sample represented a different tree.

Statistical analyses were performed with the package JMP (SAS Institute Inc.).

Results

Field plots

Attack level in field plots, measured as number of galls per shoot, was about 50% higher in trees with chestnut blight (Fig. 2). There were also differences among plots in galls per shoot, and no blight \times locality interaction (Table 1).

The presence of chestnut blight had no effects on the size, wall thickness, or toughness of *D. kuriphilus* galls (grand means \pm SD = 25 \pm 6 mm³, 3.1 \pm 0.9 cell layers, and 1272 \pm 498 g × 10 g, respectively; N = 103 galls). Larval mass was 22%

lower in plants affected by chestnut blight ($F_{1,38} = 58.11$, p < 0.0001; Fig. 3A), and there were not differences among plots (Fig. 3A). Female fecundity was strongly related to the presence of chestnut blight, the number of eggs per female was almost double in trees affected by the fungi ($F_{1,33} = 11.02$; p = 0.002; Fig. 3B). Effects of blight varied somewhat among plots (main effect of plot: $F_{3,33} = 2.26$, p = 0.084 plot × blight: $F_{3,33} = 2.32$, p < 0.077; Fig. 3B).

The presence of chestnut blight was unrelated to the nitrogen content of any of the tissues analyzed (Table 2, Fig. 4A), but there were differences among tissues and plots (Table 2, Fig. 4A). Water content was higher in galls than in leaves (Table 2, Fig. 4B), but there were no differences between trees attacked only by the insects versus trees also infected with chestnut blight (Table 2, Fig. 4B).

Secondary metabolites in field plots showed different results depending on the class of compounds. Total terpenes varied depending on the tissue (Table 3, Fig. 5A) with higher concentration in the leaf tissue surrounding the gall. There were also differences in terpene content among plots, but there were no differences between trees with- *vs.* without chestnut blight (Table 3, Fig. 5A). Differences among trees were also significant and explained about 24% of the variation. Total phenols showed similar results with more dramatic differences among the tissue analyzed (Table 3, Fig. 5B): higher phenols in ungalled leaves followed by the leaf tissue surrounding the galls, and dramatically less in the galls themselves, especially on trees affected by chestnut blight (Table 3, Fig. 5B). There were also differences among plots but no effects of the presence of chestnut blight. Concentration of condensed tannins was significantly affected by the presence of blight, tissue type, plot, and the interaction between presence of the fungus and tissue type (Table 3) with highest concentrations in the gall tissue of fungal-infected trees (Fig. 5C).

Greenhouse study

In the greenhouse study, there was a significant effect of treatment on total galls per tree ($F_{2,126} = 3.47$; p < 0.03; Fig. 6), but this was because control trees were protected from *D. kuriphilus* attack in the first year. If we only consider plants exposed to the insect, there were no differences in galls / tree in treatments with and without chestnut blight (DK+CP and DK, Fig. 6).



Figure 2. Attack level in trees with (DK + CP) and without infection by *Cryphonectria parasitica* (DK). From five field plots of chestnut trees. Figure shows galls per shoot (least square means ± SE).

Table 1. ANOVA results comparing attack levels (galls / shoot) in trees with and without chestnut blight, in each of five study plots. Corresponds to data in Fig. 2.

Source	df	F
Blight	1, 114	7.40**
Locality	4, 107	7.59***
Locality × blight	4, 107	0.86
Total buds	1, 163	238.04***
Percent random variance attributable to tree within locality × blight		51***

* p < 0.05; ** p < 0.01; *** p < 0.001

Table 2. ANOVA results comparing percent of nitrogen and percent of water of study trees with and without chestnut blight. Table shows results for three tissue types (ungalled leaves, galled leaves, and galls) from a total of 46 study trees within 5 plots that were affected by *D. kuriphilus* alone and those that were affected also by chestnut blight. Corresponds to data in Fig. 4.

S	F-statistics for nitrogen and water content					
Source	df	%Nitrogen	%Water			
Blight	1, 36	2.60	0.27			
TissueType	2, 88	18.71***	406.81***			
Blight × Tissue	2, 88	0.84	1.24			
Plot	4, 36	3.14*	2.31			
Plot × Blight	4, 36	2.57	3.46*			
Percent random variance attributable to tree within blight		43**	19			

* p < 0.05; ** p < 0.01; *** p < 0.001.



Figure 3. Mass and fecundity of *Dryocosmus kuriphilus*. From five field plots of chestnut trees, mass of late larvae and eggs / female (± SE) in trees with (DK + CP) and without (DK) infection by *Cryphonectria parasitica*. Buratai was the only plot unaffected by the late freeze of 2017. The bar labels show the number of individuals used for measurements.



Figure 4. Nitrogen and water content of leaves and galls. From five field plots of chestnut trees, percent nitrogen (**A**) and water (**B**) (\pm SE) in ungalled leaves, galled leaves, and galls of trees with and without infection by *Cryphonectria parasitica*.

Table 3. ANOVA results comparing chemical attributes of study trees with and without chestnut blight. Table shows measurements of three tissue types (ungalled leaves, galled leaves, and galls) from a total of 35 study trees and 34 study trees that were affected by *D. kuriphilus* alone, or also by chestnut blight, respectively. Corresponds to data in Fig. 5.

c.	10	F-statistics for three measures of phytochemistry				
Source	ar	Terpenes (sqrt)	Phenols (sqrt)	Tannins (sqrt)		
Blight	1, 59	0.46	1.81	61.35***		
TissueType	2, 134	4.19*	226.56***	12.20***		
Blight × Tissue	2, 134	0.51	9.81***	9.23***		
Plot	4, 59	5.59***	21.37***	8.41***		
Plot × Blight	4, 59	1.37	1.2	4.64**		
Percent random variance attributable to tree within blight		24*	6	34**		

The differences between treatments disappeared when all plants were exposed to the insect in 2019 and 2020 regardless of whether the trees were previously attacked or not (Fig. 6).

There was no difference in the number of eggs from females emerging from greenhouse plants attacked only by the insect or by both species (mean \pm SE = 131 \pm 12 and 142 \pm 15 for females from DK and DK + CP respectively).



Figure 5. Concentrations of secondary metabolites in the foliage of trees with and without chestnut blight. From five field plots of chestnut trees, concentrations of terpenes (**A**) phenolics (**B**) and tannins (**C**) in ungalled leaves, galled leaves, and galls of trees with (DK + CP) and without (DK) infection by *Cryphonectria parasitica*. Figures show means \pm SE of trees in each treatment group (square root transformed data).

There were no differences in nitrogen content due to the presence of chestnut blight or type of tissue analyzed (data not shown). Water content was also not affected by the presence of the fungus, but as in the field plots, water content was significantly higher in the galls compared with the other tissue analyzed (F $_{2,24}$ = 121.98, p < 0.0001).

The concentration of secondary metabolites in the greenhouse study differed from that measured in adult plants in the field. Terpenes were overall significantly higher in plants attacked by DK compared with control plants or plants with both invaders, although these differences disappear in the galls (Table 4, Fig. 7A). However, the presence of the fungus did not affect concentrations of phenols or tannins (Table 4, Fig. 7B, C). As in the trees of the field plots, phenol concentrations in seedlings were significantly lower in gall tissue compared with ungalled leaves or the ungalled portion of galled leaves (Table 4, Fig. 7B) and there was no effect of chestnut blight. Unlike in mature trees, there was no significant increase of tannins in seedlings infected with chestnut blight (Table 4, Fig. 7C).



Figure 6. Attacks by *Dryocosmus kuriphilus*. From potted chestnut seedlings, attacks (± SE) in plants that were, and were not, infected by chestnut blight (DK+CP and DK, respectively), and in plants that were not infected by *Cryphonectria parasitica* and were not exposed to *Dryocosmus kuriphilus* in 2018 (Control).

Discussion

Since the entry of *D. kuriphilus* into Europe, numerous associations have been described between this species and native or introduced pathogenic fungi (Seddaiu et al. 2017; Morales-Rodríguez et al. 2019). *D. kuriphilus* has been associated with a higher incidence of *C.parasitica* (Meyer et al. 2015; Vannini et al. 2018) and implicated as a potential vector of numerous other fungi (Meyer et al. 2015; Yang et al. 2021). We add to this knowledge evidence that attack rates of *D. kuriphilus* were greater in chestnut trees infected with *C. parasitica* compared with uninfected trees (Fig. 2). In the field, in plots that had been infested by *D. kuriphilus*, attack rates were about 30 - 60% higher in trees afflicted with chestnut blight compared to nearby trees with no chestnut blight (Fig. 2).

There are several potential explanations for higher attack rates in trees with chestnut blight. It might be that (1) chestnut blight promotes higher attack rates by the gall wasp. However, the greenhouse study showed that the insect does not preferentially oviposit in trees affected by the fungus. (2) It is possible that chestnut trees suffering from higher attack densities by the gall wasp are made more susceptible to chestnut blight. Some previous studies show that C. parasitica may benefit from D. kuriphilus, since galls are a potential source of fungal inoculum (Meyer et al. 2015). Vannini et al. (2018) also described an increase of fungal infection in the crown of chestnuts related with D. kuriphilus attack. However, the symptoms of C. parasitica in our study trees were large cankers in the stems that almost certainly preceded the arrival of D. kuriphilus. (3) There were more attacks on trees with blight because the female adults emerging from these trees had twice the fecundity of those emerging from uninfected trees (Fig. 3B) and females frequently oviposit in the same tree from which they emerged (Castedo-Dorado et al. 2023b). This hypothesis predicts high philopatry in D. kuriphilus. (4) It is possible that there is a genetic association between susceptibility to blight and oviposition preferences of D. kuriphilus. Such an association was not evident in our greenhouse studies with saplings, but the expression of chestnut blight in adult trees in nature is more likely to reflect genetic susceptibility than occurrence of blight in



Figure 7. Concentration of secondary metabolites in potted chestnut seedlings. Concentrations of terpenes (A), phenolics (B), and tannins (C) in the ungalled leaves, galled leaves, and galls of plants that were and were not infected by chestnut blight (DK + CP and DK, respectively). Figures show means \pm SE of trees in each treatment group (square root transformed data).

Table 4. ANOVA results comparing chemical attributes of study trees from the greenhouse seedlings. Table shows results of three tissue types (ungalled leaves, galled leaves, and galls) from a total of 43 study trees that were also affected by *D. kuriphilus* and those that were affected also by chestnut blight. Corresponds to data in Fig. 7.

£	df	F-statistics for three measures of phytochemistry				
Source		Terpenes (sqrt)	Phenolics (sqrt)	Tannins (sqrt)		
Blight	1,17	9.42**	2.9	1.44		
TissueType	2, 34	1.02	19.47***	1.68		
Blight × Tissue	2, 34	1.9	3.34*	0.43		
Percent random variance attributable to trees within blight		0	26	5		
* p < 0.05; ** p < 0.01; *** p < 0.001.						

saplings. Hypotheses 3 and 4 are not mutually exclusive. Further studies will be necessary to discriminate among these hypotheses.

Larval mass was lower in trees affected by chestnut blight (Fig. 3A). However, these differences were apparently not due to nutritional quality because there were no differences in nitrogen or water content of gall tissue, where the larvae develop (Fig. 4). The effects of chestnut blight on *D. kuriphilus* fecundity were also dramatic but in the opposite direction (> 2-fold higher in some the plots; Fig. 3B). This surprising result suggests that larvae were growing at similar rates in both tree types, but that larvae grew for a longer time, and therefore became bigger adults, in trees with chestnut blight.

Plant chemistry offers potential explanations for the higher fecundity of *D. ku-riphilus* in trees with chestnut blight. Higher fecundity in trees with chestnut blight was not due to nutritional quality because there were no differences N concentration in galls (Fig. 4A). Higher fecundity in trees with chestnut blight could be due to reduced chemical defenses in the plant tissue (Abrahamson et al. 2003; Naidoo et al. 2018), but the evidence argues against this hypothesis. Neither terpenes nor phenols were in general lower in trees afflicted with chestnut blight compared to those that did not have chestnut blight (Fig. 5). Phenolics were lower in the galls of trees with chestnut blight (Fig. 5B), but this could be a result of concomitant increases of tannins (Fig. 5C).

The most remarkable phytochemical difference between trees with and without chestnut blight was the high concentration of condensed tannins in galls (Fig. 5C). Virtually, all trees affected by *C. parasitica* (97%) showed measurable amounts of tannins within galls, while less than half (37%) of trees affected only by the insect showed measurable tannins within galls. Tannins are part of the antifungal and antimicrobial defense systems in many plant species (Uchida 1977; Griffin and Elkins 1986). Increased tannin concentration in the host can often be seen directly surrounding mycelial mats (Lovat and Donnelly 2019). Some studies have suggested that accumulation of tannins is related to resistance to chestnut blight (Nienstaedt 1953; McCarroll and Thor 1985; Gao and Shain 1995; but see Anagnostakis 1992). However, other studies have suggested that host tannins are used as a carbon source by the fungus through the activity of tannase produced by *C. parasitica* (Cook and Wilson 1915; Elkins et al. 1979). The role of tannins and tannase in chestnut blight pathology remains to be defined (Lovat and Donnelly 2019).

A higher concentration of tannins might influence wasp abundance and attack rates in the field. Castedo-Dorado et al. (2023b) suggested that, in the early stage of invasion, a substantial proportion of adults re-infect the same tree where they developed. Cornell (1983) suggested that high tannin concentrations in galls serve as a protective barrier for larvae developing inside the galls against fungi and other herbivores that are unable to induce galls, but feed on them. Tannins may protect galls from fungal infestation (Taper and Case 1987). Fungal damage is common in galls of *D. kuriphilus* especially caused by *Gnomoniopsis castaneae* (Magro et al. 2010; Maresi et al. 2013; Lione et al. 2015; Muñoz-Adalia et al. 2019). The pathogen has been associated with increased mortality of emerging adults (Magro et al. 2010; Vannini et al. 2014, 2017). The protection generated by tannins could help reduce the impact of this pathogen on *D. kuriphilus* populations developing in trees attacked by chestnut blight. The protection generated by tannins could be an adaptive explanation for why larvae in trees with chestnut blight grew for a longer time and thereby attained higher adult fecundity.

The association between chestnut blight and elevated tannins was not evident in the greenhouse study with seedlings (Fig. 7C) and there was no effect of chestnut blight presence in female fecundity. This may be attributed to the recent occurrence of fungal attack since these seedlings were infected by the disease in the same spring, from contamination during grafting. It could also be related to differences in physiology between seedlings and trees. In any case, trees afflicted with chestnut blight over some years, as in the typical course of chestnut blight infection, had elevated tannin levels in the galls, and the insects that developed within those galls had notably higher fecundity.

Tannins have commonly been regarded as anti-herbivore defenses (Donaldson and Lindroth 2004; Barbehenn and Constabel 2011) which may affect insect fecundity. But if this were the case in our system, we would have expected lower fecundity, rather than higher, in trees that had chestnut blight and displayed higher tannin concentrations in gall tissue (Fig. 5C). Adapted herbivorous insects may benefit from the presence of tannins in their food plants (Karowe 1989) and a variety of tree-feeding Lepidoptera are stimulated to feed by tannic acid (Bernays 1981). Rehill and Schultz (2012) found a positive relationship between condensed tannin concentration and the reproductive performance of fundatrices in another gall maker.

The higher fecundity of D. kuriphilus in trees with chestnut blight could also be related to the environment provided by the galls themselves. Arriola et al. (2018), working with another gall maker, showed that galls provided protection but did not enrich nutrition. Galls may protect the insect within from unfavorable abiotic conditions, particularly desiccation (Microenviroment Hypothesis; Price et al. 1987; Miller et al. 2009). However, the presence of chestnut blight had no effects on the size, wall thickness, or toughness of D. kuriphilus galls. Nor were there effects of chestnut blight on the water content of tissue within galls (Fig. 4). Still, Lombardero et al. (2021), working in the same plots with trees attacked by D. kuriphilus but not by chestnut blight, found greatly reduced fecundity in 2018 in populations exposed to a late freeze in the spring of 2017. All plots in the current study (except Buratai) were affected by the same freezing event, and in the next year (2018), all plots except Buratai, showed much higher fecundity in galls that developed in trees with chestnut blight compared to trees without chestnut blight (Fig. 3B). This could be understood if higher concentration of tannins in gall tissue helped to protect part of the insect population from low temperatures. Tannins can have quite general effects in protecting plant tissue from abiotic stress (Dehghanian et al. 2022) and may contribute to a physical barrier that isolates the gall insect from external environmental conditions. Uhler (1951) suggested that galls may protect against sudden changes in temperature. One prediction is that the thermal insulation within tannin-rich galls is greater when the galls are particularly rich in tannins, as in chestnut trees afflicted with chestnut blight.

Conclusion

The introduction of invasive species can give rise to novel community interactions, and sometimes new positive associations among plant enemies. Our results indicate that the presence of chestnut blight increases the suitability of chestnut trees for the invasive galling insect, *D. kuriphilus*. Potential explanations include ameliorating the negative effects of environmental conditions. In any case, the positive association between chestnut blight and chestnut gall wasps suggests that management efforts to limit the incidence of chestnut blight may have additional benefits in reducing damage from the chestnut gall wasp.

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: MJL. Data curation: MJL, MFRR. Formal analysis: MFRR, MPA, MJL. Funding acquisition: FCD, MPA, MJL. Methodology: FCD, MPA, ANP, MFRR, MJL. Visualization: MJL, FCD. Writing - original draft: MFRR. Writing - review and editing: MPA, MJL, ANP, FCD.

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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

Thematic mapping of biosecurity highlights divergent conceptual foundations in human, animal, plant and ecosystem health

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Abstract

Effective biosecurity policies are essential to address several major sociological and environmental challenges facing humankind including existential pandemic risks, threats to food security, loss of ecosystem services and public resistance to pesticides and vaccines. Yet biosecurity is subject to multiple interpretations that include dealing with the threats from bioterrorism, managing laboratory biosafety to prevent the escape of pathogenic organisms, handling food and agricultural production systems to prevent disease introduction and addressing the threat of introduced organisms to flora, fauna and humans. The absence of a shared vision of what biosecurity encompasses means that decision-makers are often challenged to design appropriate biosecurity policies at national and global scales. The design of effective policy strategies requires an understanding of the methodological and conceptual barriers that constrain attempts to build an interdisciplinary approach to biosecurity. Here, the first thematic map of the biosecurity research landscape is undertaken to assess just how diverse the interpretation of biosecurity is amongst the global research community and the extent to which the articles published since 2000 represent a common conceptual foundation or are largely clustered within sectors. Co-citation, bibliographic coupling and co-word analyses highlighted that the field of biosecurity encompasses a wide range of domains from biochemistry through to political science, but the research supporting different sectors largely draws from a distinct literature base. While ecosystem and plant health were clustered together within the broad grouping of biological invasions, there was a clear separation from both human and animal health. Yet, there is considerable scope for the management of biological invasions to benefit from insights derived from social perspectives in human and animal health. Biosecurity remains divided by conceptual differences and specialised vocabularies that limit the effectiveness of biosecurity policies addressing biodiversity conservation, public health and food security. To overcome these constraints requires the building of a global biosecurity community that accepts a broader definition of biosecurity, avoids sectorial jargon and establishes mechanisms to cultivate interdisciplinarity through specialised collaborative centres, cross-sectorial research programmes and conceptually rich training programmes.

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Copyright: © Philip E. Hulme. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). **Key words:** Epidemiology, invasive alien species, One Biosecurity, One Health, pests, social network analysis, surveillance, zoonoses

Introduction

The term biosecurity has become increasingly popular within the scientific literature in the last two decades pointing to a greater focus on this topic, particularly following the SARS-CoV-2 pandemic in 2019 (Fig. 1). Despite the increasing use of this term, multiple definitions of biosecurity can be found in standard reference texts (Table 1). Biosecurity is variously defined in terms of dealing with the threats



Figure 1. Temporal trend in the number of articles archived in Web of Science and published between 2000 and 2022 that include biological invasion, biosafety, biosecurity or bioterror (including bioterrorism and bioterrorist) in the article title, author keywords or Keywords Plus. The figure illustrates the relative growth in interest in biosecurity compared to the other terms over the last two decades.

Table 7	1 . Definitions	of the term	biosecurity as	published	in several	Oxford	dictionaries	by Oxford	University	Press.	The name	of the
dictiona	ary and its desc	cription of bi	osecurity are j	provided.								

Oxford dictionary	Description
Biomedicine (Lackie and Nation 2019)	Describing methods or procedures designed to prevent harm from pathogenic organisms that are being handled for experimental purposes.
Agriculture & Land Management (Manley et al. 2019)	A series of planned measures introduced to a farm or enterprise concerned with food production that minimises the risk of accidental disease introduction.
Human Geography (Rogers et al. 2013)	The security of a country's human population, flora and fauna against the unwanted introduction of various biological phenomena (such as viruses, toxins, insects, plant species and mammals).
Environment and Conservation (Park and Allaby 2017)	Biological security, particularly protection against bioterrorism and the use of biological weapons.
Geography (Mayhew 2023)	The protection of people and animals from pests and infectious diseases, notably by managing the movement of agricultural pests and diseases, reducing the effects of invasive species on supposedly indigenous flora and fauna and preventing the purposeful and inadvertent spreading of biological agents into the human population.
Advanced Learner's (Hornby 2010)	The activities involved in preventing the spread of animal and plant diseases from one area to another.
Concise English (Stevenson and Waite 2011)	Measures taken to protect the population against harmful biological or biochemical substances.
English (oed.com)	Protection against the incursion or escape of potentially harmful or undesirable organisms, especially pathogens.
New Zealand English (Deverson and Kennedy 2005)	Procedures followed or measures taken to safeguard the flora and fauna of a country etc. against exotic pests and diseases.
American English (Stevenson and Lindberg 2010)	Procedures intended to protect humans or animals against disease or harmful biological agents.

from bioterrorism, managing laboratory biosafety to prevent the escape of pathogenic organisms, handling food and agricultural production systems to prevent disease introduction and addressing the threat of introduced organisms to flora, fauna and humans. The confusion as to the definition of biosecurity also exists at an international level. Even within the United Nations (UN), separate specialised agencies that have oversight of pest and/or disease outbreaks use different definitions. The UN Food and Agriculture Organisation defines biosecurity as a strategic and integrated approach to analysing and managing relevant risks to human, animal and plant life and health and associated risks to the environment (FAO 2007). In contrast, the World Health Organisation views biosecurity as synonymous with biosafety and it is limited to approaches to prevent the unauthorised access, loss, theft, misuse, diversion or release of pathogenic biological agents (WHO 2020). The World Organisation for Animal Health has a more specific definition of biosecurity as a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections or infestations to, from and within an animal population (Renault et al. 2021). This lack of a consistent definition may be the reason that, in its guidance for negotiators of multilateral environmental agreements, the United Nations Environment Programme provides definitions for biosafety and alien species, but not for biosecurity (UNEP 2007).

The lack of a standard definition of biosecurity is much more than simply an etymological problem. Without a shared vision for what biosecurity encompasses, decision-makers are challenged to design appropriate biosecurity policies at national and global scales (Meyerson and Reaser 2003). There are clearly different perspectives amongst sectors (e.g. agricultural, environmental, medical, military) that will lead to divisions amongst those who view biosecurity as a laboratory biosafety issue, a means to combat bioterrorist threats, an approach to better manage farm hygiene, tools to prevent incursions of pests and diseases harmful to agriculture or measures to mitigate a major driver of biodiversity loss and ecosystem change. This diversity of views can lead to a fragmented approach to biosecurity in terms of policy coverage, education and training, as well as research investment. The absence of a consensus view is also a major stumbling block for any attempts to establish an overarching global One Biosecurity convention that would simultaneously address biological invasions that threaten human, animal, plant and ecosystem health (Hulme 2021).

While attempts to derive a universal definition of biosecurity are certainly worthwhile (Meyerson and Reaser 2002b), such a top-down approach may fail to account for sectorial differences in how biosecurity is perceived and implemented amongst the diverse human, animal, plant and ecosystem health research community. Progressing consensus within the research community as to what biosecurity means is a crucial step in delivering a cohesive evidence base that supports policy-makers to deliver better integrated biosecurity policies (Hulme 2021). Consensus is also essential to address several major sociological and environmental challenges impacting on biosecurity, such as climate change, increasing urbanisation, agricultural intensification, human global mobility, loss of technical capability as well as public resistance to pesticides and vaccines (Hulme 2020).

The design of effective policy strategies requires an understanding of the methodological and conceptual barriers that constrain attempts to build an interdisciplinary approach to biosecurity and these obstacles are strongly connected to the nature of scientific practice and the limits of human cognition (MacLeod 2018). Knowledge of the scientific landscape, its conceptual foundations, emerging ideas and vocabulary can provide insights into these cognitive barriers. Here, the first scientific map (Chen 2017; Chen and Song 2019) of biosecurity is undertaken to assess just how diverse the interpretation of biosecurity is amongst the global research community and the extent to which the science represents a common corpus of knowledge or is largely clustered within sector specific siloes. The analysis aimed to:

- 1. Capture the breadth of disciplines associated with biosecurity research.
- 2. Characterise the dominant conceptual themes examined within the research corpus.
- 3. Describe the extent to which the research is coupled across different sectors.
- 4. Identify the cognitive barriers preventing an interdisciplinary approach to biosecurity.

These findings are then used to explore how research underpinning biosecurity can be made more interdisciplinary and identify unifying principles that are common to different disciplines, but where there is currently little cross-fertilisation.

Methods

Data retrieval

Bibliographic data were extracted from the Web of Science Core Collection of Citation Indexes (which includes science, social sciences, arts and humanities citation indexes) for research articles, reviews, conference proceedings, book chapters, editorial material and letters relating to the single search term - biosecurity - published during the 23-year period from January 2000 to December 2022 inclusive (accessed on 6 June 2023). To ensure the search only extracted the most relevant articles, the fields mined in the search were restricted to the title of the article, author-defined keywords and Keywords Plus. Keywords Plus is a set of keywords automatically generated by Web of Science from the cited bibliography that is claimed to capture an article's content with greater depth and clarity than author-generated keywords (Garfield and Sher 1993). The use of author-defined keywords together with Keywords Plus is an effective way to capture the knowledge structure of scientific fields as well as an article's content (Zhang et al. 2016; Hulme 2022). The full record, including cited references, was downloaded for each article retrieved and imported into the specialist software tool VosViewer 1.6.19 for constructing and visualising bibliometric networks (van Eck and Waltman 2010; van Eck and Waltman 2014).

Thematic mapping

The Web of Science assigns each journal, book or conference proceedings to one or more of 252 Research Areas (hereafter described as WoS Research Areas) in science, social sciences, arts and humanities and is generally considered the best high-level classification scheme for detailed bibliometric analysis (Milojević 2020). While many bibliometric studies simply tally the most frequently represented research areas, such an approach fails to capture levels of interdisciplinarity since many journals are assigned to multiple categories. Thus, for a more effective high level thematic map, the co-occurrence of different WoS Research Areas assigned to individual articles was visualised as a network to distinguish distinct thematic areas.

Although there are different ways to assess the connectivity with a corpus of research articles (Enders et al. 2019; Staples et al. 2019; Muñoz-Mas et al. 2021), a comparison of co-citation and bibliographic coupling provides an effective means to map the underlying relational structure amongst research articles (Donthu et al. 2021). Co-citation analysis quantifies the frequency with which two different articles are both independently cited by one or more articles, such that the more publications that cite these two articles, the stronger their co-citation (Small 1973). Bibliographic coupling measures how often two articles cite the same third article indicating that they may cover the same topic and the more common references they share, the stronger their coupling strength (Jarneving 2007). These two methods provide different, but complimentary insights into the thematic structure of scientific literature since co-citation describes the linkages amongst cited publications to understand how the main themes in a research field have developed, whereas bibliographic coupling describes the relationships amongst citing publications to provide insights into current development of the field (Boyack and Klavans 2010; Kleminski et al. 2022).

Co-word analysis examines the co-occurrences of keywords to explore the existing relationships amongst topics in a research field by focusing on the written content of the publication itself to build a conceptual structure of the domain (Donthu et al. 2021). This semantic map helps to understand the cognitive structure of a research field, but assumes that the concepts behind co-occurring words are closely related (Zupic and Čater 2015). Both author-defined keywords and those generated by the Keywords Plus algorithm were extracted from each article retrieved and assumed to be the key description of its research content. The list of keywords was reviewed and simplified by standardising across synonyms, abbreviations and acronyms as well as alternative spellings of words. Generic terms such as science, biology, program etc. were ignored as were keywords relating to a specific country or region. Finally, given that the initial search term was biosecurity, this word was excluded from the co-word analysis.

Visualisation

In the cases of both co-citation and bibliographic coupling, only those articles that had received at least 10 citations were included in the analyses to avoid arbitrary clustering associated with infrequently cited articles. Fractional-, rather than full-counting was implemented since it has been shown to produce better balance and consistency in bibliometric indicators by reducing the influence of highly-cited articles and those with large bibliographies (Perianes-Rodriguez et al. 2016; Szomszor et al. 2021). To ensure dominant themes were captured, co-word analysis included only keywords that occurred five or more times and full-counting was implemented. For the analysis of WoS Research Areas, all articles were included and full-counting was implemented. For all four analyses (WoS Research Areas, co-citation, bibliographic coupling and co-word analysis), association strength was selected as a probabilistic-based similarity measure to normalise the maps for visualisation. The association strength compares the number of observed co-occurrences between two entities (e.g. articles, keywords or WoS Research Areas) against the null expectation of co-occurrences being randomly distributed to give an indication how strong the relationship is between a pair of entities (Steijn 2021). Association strength is particularly suited for bibliometric analysis and has the advantage of detecting highly-cited articles regardless of citation traditions in different disciplines (van Eck and Waltman 2009). The total strength of the citation links of a given article with other publications (total link strength or weighted degree) was applied as a weight. Articles were clustered using a smart local moving algorithm to

identify community structures with higher modularity values (Waltman and van Eck 2013) and depicted using the visualisation of similarities distance-based mapping technique that has been shown to produce better structured thematic maps than alternatives such as multidimensional scaling (van Eck and Waltman 2010).

Network metrics

There are many different measures of network centrality that provide an indication of the relative importance of a particular node within a network, but they are rarely used in an a priori approach to describe different types of networks (Rodrigues 2019). Three different measures of network centrality were used to capture the relative importance of WoS Research Areas, citation (whether co-citation or bibliographic coupling) and co-word networks. Betweenness centrality was selected as the best measure of WoS Research Area importance since it identifies nodes that act as bridges across different parts of the entire network and hence a WoS Research Area with high betweenness centrality is likely to be the most interdisciplinary and thus crucial to the interconnectedness of a network (Leydesdorff 2007). In contrast, eigenvector centrality was used to identify important articles in both co-citation and bibliographic coupling networks since it provides a measure of node influence since articles with high eigenvector centrality will themselves be linked to other articles that have many connections (Diallo et al. 2016). Finally, the most important keywords were identified as those with the highest harmonic closeness centrality since this indicates which words are most closely associated with other words and, thus, likely to be most representative (Choudhury and Uddin 2016). Centralities measures were calculated using the Gephi 0.1 network software (Bastian et al. 2009).

Results

A total of 3,685 articles were retrieved by the title, author-defined keyword and Keywords Plus search for the term biosecurity. This corpus encompassed 115 out of a possible 252 WoS Research Areas, but the vast majority (91.6%) of the retrieved articles were captured by only 23 WoS Research Areas. The primary WoS Research Areas as determined by the number of articles retrieved were Veterinary Sciences (1,011 articles), Environmental Science & Ecology (540), Agriculture (483), Biodiversity & Conservation (257), Public, Environment & Occupational Health (222) and Science & Technology (204). These top 23 WoS Research Areas described five marked clusters (Fig. 2) that map on to animal health (e.g. Veterinary Sciences, Infectious Diseases), plant health (e.g. Agriculture, Food Security and Plant Science), ecosystem health (e.g. Environmental Sciences, Biodiversity and Conservation), a socioeconomic cluster (e.g. Public Health, Social Sciences, Government and Law) and a technology cluster (e.g. Science and Engineering). Environmental Science & Ecology was the WoS Research Area with by far the highest betweenness centrality (0.238), followed by Veterinary Sciences (0.095), while Engineering (0.00) was the least important and was relatively isolated from other nodes (Fig. 2).

Almost one quarter (841 or 22.8%) of the articles cited by the retrieved literature were co-cited at least 10 times, with the earliest article being published in 1921 (Montgomery 1921). The co-citation network comprised 37,770 edges linking the 841 nodes and was strongly modular with four distinct clusters identified (Fig. 3a). The largest cluster (364 articles) comprised articles addressing biological



Figure 2. Thematic map of 23 Web of Science Research Areas that encompass over 90% of all articles in the biosecurity corpus. The relative number of publications that fall under each WoS Research Area (size of circles) and the strength of the links between them (line thickness) are displayed. Five clusters can be identified and are highlighted by the ellipses.

invasions relating to both plant and ecosystem health with the top three journals being Biological Invasions, Trends in Ecology & Evolution and Frontiers in Ecology and Environment. The second cluster (331 articles) included primarily articles within the field of animal health as described by the top three most frequently occurring journals (Preventive Veterinary Medicine, Transboundary and Emerging Diseases and Veterinary Clinics of North America: Food Animal Practice). Socioeconomic perspectives were represented by the third cluster (104 articles) with an emphasis on rural livelihoods relating to farming as seen in the top three journals: Environment and Planning A: Economy and Space, Sociologia Ruralis and Transactions of the Institute of British Geographers. The smallest cluster (42) dealing with human health in relation to pathogens and biosafety as published in Nature, Science and World Health Organisation reports. The five most influential articles, as determined by their eigenvector centrality were all related to aspect of biological invasions whether specifically addressing the biosecurity system and its components (Meyerson and Reaser 2002a; Waage and Mumford 2008) or more specifically in terms of the role of trade (Hulme 2009), introduction pathways (Hulme et al. 2008) and economic costs (Pimentel et al. 2005).

Over one of third of articles met the criteria for bibliographic coupling (1,359 or 36.87%) with 43,490 links between them. The network analysis revealed broadly similar trends as for co-citations, but with greater granularity revealing ten rather than four distinct article clusters (Fig. 3b). Three clusters were strongly aligned to animal health, with differences amongst them relating to the livestock concerned whether cattle and pigs, poultry or horses. Preventive Veterinary Medicine was amongst the top three most frequently occurring journals in each of these three



Figure 3. Visualisation of: **a** co-citation and **b** bibliographic coupling of the corpus of articles retrieved using search term biosecurity in titles, author keywords or Keywords Plus published between 2000 and 2022. The two analyses share a similar topology with four clusters identified in co-citation analysis and ten in the bibliographic coupling. Each cluster has been given a representative description and the number of articles in each cluster are presented within parentheses.

clusters. A further three clusters were aligned to pest, pathogen and weed invasions in both semi-natural and agricultural ecosystems. Amongst these, the largest cluster related primarily to the theme of biological invasions with over 10% of the articles being published in Biological Invasions, while the three other clusters were characterised by studies of introduction pathways (e.g. Management of Biological Invasions), plant pathology (e.g. Plant Pathology) and molecular diagnostics (e.g. Molecular Ecology Resources). A discrete human perspectives cluster was also still evident that reflected a similar composition to the co-citation analysis with journals dealing with rural studies being prominent (e.g. Environment and Planning A: Economy and Space, Journal of Rural Studies). Rather than being directly associated with human health, a further cluster was comprised largely of articles in journals that focused on health security and bioterrorism (e.g. Frontiers in Bioengineering and Biotechnology). The final cluster appeared to bridge across the animal health and biological invasions clusters and mostly encompassed journals addressing aquaculture both from a production perspective, but also in terms of risks of invasion (e.g. Aquaculture, Journal of the World Aquaculture Society). The five most influential articles as determined by their eigenvector centrality were reviews that addressed human perceptions (Cliff and Campbell 2012), communication (Hanrahan and Melly 2019) and awareness (Klapwijk et al. 2016) of invasive alien species or general syntheses of biological invasions (Russell et al. 2017; Cope et al. 2019).

Across both author-defined keywords and those generated by the Keywords Plus algorithm, a total of 5,804 distinct terms were retrieved, of which 308 occurred sufficiently frequently (more than five times) to be analysed further (Fig. 4). Four clusters of terms were identified with once again a clear distinction between terms characterising animal health (e.g. infection, transmission, epidemiology) from those that related to biological invasions (e.g. surveillance, spread, invasive alien species). A further discrete cluster related to terms describing emerging human health threats from pathogens (e.g. disease, virus, infectious disease). A final cluster captured human responses to biosecurity threats and, as a result, spanned both the animal health and biological invasions cluster since the most central terms in this cluster were relevant to both areas as well (e.g. management, prevention, policy).

Clear differences were found in the most frequent keywords associated with the four clusters (Table 2). The biological invasions cluster is strongly shaped by terms associated with the invasion continuum (e.g. spread, impact), major pathways of

Count	Biological invasions	Count	Animal health	Count	Human risks	Count	Response	
360	invasive alien species	303	pigs	320	320 virus		management	
284	biological invasion	269	poultry	311	disease	142	farmer	
211	surveillance	260	cattle	130	biosafety	132	prevention	
190	impact	237	transmission	118	biowarfare	116	veterinary	
165	spread	220	infection	84	infectious disease	111	policy	
140	eradication	189	avian influenza	80	covid-19	108	perceptions	
126	trade	157	epidemiology	63	public health	100	risk management	
123	risk assessment	138	antimicrobial resistance	59	agriculture	90	knowledge	
109	pathogens	131	outbreak	58	influenza	88	attitude	
105	climate change	123	foot and mouth disease	53	dual use research	86	behaviour	
101	polymerase chain reaction	118	dairy	39	One Health	72	strategies	
100	quarantine	114	vaccination	34	food	64	decision-making	
96	biodiversity	104	salmonella	33	challenges	61	animal health	
94	identification	103	farm	33	human	57	bovine tuberculosis	
89	plant disease	95	herds	31	global health security	46	animal disease	
88	risk analysis	82	equine	30	pandemic	41	communication	
87	dispersal	78	livestock	25	globalisation	38	environment	
79	pathway	71	epidemic	22	preparedness	38	information	
77	pests	67	disinfection	19	ebola	38	uncertainty	
76	threat	65	zoonoses	15	capacity building	29	stakeholder	
70	populations	64	Escherichia coli	14	husbandry	23	wildlife	
67	transport	63	respiratory disease	14	migration	22	participation	
65	conservation	62	African swine fever	11	cyberbiosecurity	21	badgers	
57	aquaculture	62	campylobacter	11	swine influenza	18	awareness raising	
53	costs	55	bacteria	10	bat	16	compliance	

Table 2. The 25 most frequent keywords in each of the four clusters identified through co-word analysis: biological invasions, animal health, human risks and response.



Figure 4. Co-word analysis of 308 terms derived from Keywords Plus that were cited more than five times in articles from the biosecurity corpus published between 2000 and 2022. Four clusters can be identified that relate to animal health (e.g. infection, transmission, epidemiology), biological invasions (e.g. surveillance, spread, invasive alien species), human risks from emerging threats from pathogens (e.g. disease, virus, infectious disease) and the response to biosecurity threats (e.g. management, prevention, policy).

introduction (e.g. pathway, trade, transport, aquaculture) and management (e.g. surveillance, eradication, risk assessment). In contrast, the animal health cluster has a strong representation of the target livestock (pigs, poultry, cattle, equine) and livestock diseases (e.g. foot and mouth disease, African swine fever, avian influenza), as well as management tools (e.g. vaccination, epidemiology, disinfection). The human risks cluster described emerging threats from human activities (e.g. biowarfare, biosafety, dual use research), high profile zoonotic diseases (e.g. Covid-19, Ebola, influenza) and society's response (e.g. public health, One Health, global health security). The response cluster included keywords relating to human perspectives (e.g. perceptions, attitude, behaviour), the importance of engagement (e.g. awareness raising, communication, participation) and subsequent actions (e.g. decision-making, policy, strategies). The association of this cluster with terms, such as veterinary, animal disease and farmer, point to this area being most strongly developed for animal health.

Discussion

Biosecurity is clearly an interdisciplinary subject that encompasses human, animal, plant and ecosystem health, but also requires the involvement of economists, epidemiologists, engineers, policy-makers, public health specialists, social scientists and taxonomists. While this breadth of coverage and underpinning expertise is a strength that underlies the importance of biosecurity to society, the economy and the environment, it is also a weakness that results in multiple interpretations of its core definition. Indeed, many sectors interpret biosecurity only in terms of their own priorities and needs, whether it is in relation to dual use research, quarantine regulations or farm hygiene. As a result, the research landscape is fragmented with the consequence that knowledge is not shared widely and, thus, often fails to bring a sufficient critical mass of expertise to bear upon fundamental aspects of biosecurity that are common to human, animal, plant and ecosystem health (Hulme et al. 2023). Bibliometric analysis is particularly well suited to the analysis of a corpus of research publications and, while the articles in the biosecurity corpus included house journals of policy-making organisations (e.g. EFSA Journal, Revue Scientifique et Technique-Office International des Epizooties), it did not comprehensively review global, regional, national and sector policy documents. While it might be expected that there is cross-fertilisation in terminology across research and policy, the explicit exploration of terminology amongst policy-makers in the future would be valuable. Nevertheless, the comparison of co-citation, bibliographic coupling and co-word analysis provides a unique opportunity to assess the cognitive barriers that are obstacles to an interdisciplinary approach to biosecurity.

Co-citation analysis examines a corpus of articles in terms of its most highly-cited articles and, thus, provides a basis to understand the fundamental aspects of a research field. The co-citation analysis revealed marked segregation amongst research themes that addressed biosecurity, illustrating that the research supporting different sectors largely draws from a distinct literature base. While ecosystem and plant health were clustered together within the broad grouping of biological invasions, there was a clear separation from both human and animal health. Since co-citations reflect the frequency with which two articles are cited together in other documents, the strong clustering and segregation highlights that there is little crossover in the biosecurity relevant literature cited in the study of biological invasions, human health or animal health. Those articles in the human health cluster that had greatest affinity for the biological invasions cluster addressed issues of agro- or bioterrorism (Meyerson and Reaser 2002b; Wheelis et al. 2002), while those with affinity to the animal health cluster addressed generic issues regarding the implementation of biosecurity strategies (FAO 2007; Oidtmann et al. 2011). In addition to the segregation of human health, animal health and biological invasions, articles addressing social perspectives were also separated from the other three clusters. Nevertheless, the social perspectives cluster was more closely aligned to human and animal health than biological invasions and included articles criticising the "emerging disease worldview" of multinational organisations (King 2002) and the governance of biosecurity particularly in relation to animal health (Donaldson 2008; Enticott 2008). The integration of social science perspectives into the study of biological invasions is critical (Ricciardi et al. 2017; Shackleton et al. 2019), but the co-citation

analysis indicates that there is much further to go to achieve this goal. While some articles in the co-citation analysis integrate biological, epidemiological and policy responses to invasive alien species outbreaks (Potter et al. 2011), there is considerable scope for learning from the social perspectives in both human and animal health to better manage biological invasions.

By considering recently-published research that has fewer citations, bibliographic coupling highlights articles that share a common conceptual background and the more references in common, the stronger their connection in a bibliographic network (Zupic and Čater 2015). As it represents recent knowledge, bibliographic coupling highlights articles that are often overlooked in co-citation analysis. Despite this distinction in analytical approach, the results of bibliographic coupling for the corpus of articles addressing biosecurity revealed similar trends as those found for co-citation. The network topology reflected a similar pyramid structure that segregated studies addressing biological invasions (incorporating ecosystem and plant health) from animal health and human health with social perspectives playing a more central role. However, the analysis highlighted subclusters within both the biological invasions and animal health clusters. This might indicate that research is becoming more, rather than less, fragmented in recent years and increasingly draws from a distinct literature base. Within the topic of biological invasions, there were distinct subclusters relating to plant health, pathways and diagnostics while, for the animal health cluster, there were subclusters associated with different animal production systems. Biosecurity issues arising from aquaculture appear to bridge the animal health and biological invasions clusters due to the concerns that pathogens will spread from aquaculture into natural environment and infect wildlife (Bouwmeester et al. 2021; Bray et al. 2024). Social perspectives remain integral to both the human and animal health clusters and there is also evidence that the field of biological invasions has begun to embrace this aspect in the last decade (Tassin and Kull 2015; Head 2017).

Segregation of the human, animal, plant and ecosystem health aspects of biosecurity was also evident when examining keywords. This is in part because of the taxonomic differences between both the biosecurity threats (e.g. plants, microbes, animals) and the recipient target (e.g. humans, livestock, plants). Framing research article keywords along taxonomic lines is clearly valuable to share information within specific topics, particularly for pathogens, but can create a barrier for interdisciplinary communication (Paap et al. 2020). A more effective way to increase interdisciplinary communication across the wide range of biosecurity topics would be to focus on the fundamental processes of species introduction, establishment, spread and impact, as well as the associated responses, such as surveillance, monitoring and control (Hulme 2020). There are clear parallels between the entry and spread of an invasive alien species with the establishment of an epidemic pathogen (Nuñez et al. 2020; Vilà et al. 2021). The challenge is that, even though the process shares many parallels, the terminology used can differ, for example, virulence vs. invasiveness, transmission vs. spread, outbreak vs. incursion, epidemic vs. invasion. In addition, the same term, such as "vector", may be visualised quite differently by invasion biologists (e.g. cargo freighter) and veterinarians (e.g. mosquito) despite the fact that a common definition that encompasses both these perspectives is widely recognised as "any living or non-living carrier that transports living organisms intentionally or unintentionally" (ICES 2005). While comprehensibility is often a challenge to the

collaboration between scientific disciplines within the sectors charged with biosecurity, the different terminology used has sufficient equivalence that scope exists to communicate ideas across all sectors, but only if researchers and policy-makers are prepared to make the effort.

Going forward, there are at least three steps that need to be taken to increase interdisciplinarity within biosecurity. The first, of course, is to develop an agreed definition that encompasses the diverse perspectives of biosecurity since the multiple definitions used today act to entrench research and policy within narrow confines. One such definition could be "the research, procedures and policies that cover the exclusion, eradication or effective management of the risks posed by the introduction of alien plant pests, animal pests and diseases, animal diseases capable of transmission to humans (zoonoses), the release of genetically modified organisms and their products and the management of invasive alien species and genotypes" (Hulme 2020). A common definition will aid researchers and policy-makers understand that, while their own ambit may be focused only on human, animal, plant or ecosystem health, their work contributes to a much wider goal and that lessons can be learnt from other aligned sectors. This is a critical step in developing a global biosecurity community.

The second is to realise that even with the broad definition of biosecurity, scientific practices are often domain specific, which helps researchers solve complex problems in a cognitively manageable way (MacLeod 2018). Yet, increasingly, there is a convergence in underpinning methodologies to support biosecurity. For example, the application of environmental DNA and RNA for tracing species, the opportunity to apply new technologies for remote surveillance of human, animal, plant and ecosystem health and their associated threats, state-of-the-art decision support tools that provide information for the cost-benefits of different management strategies and social-science approaches that can lead to behaviour change and/or better engagement with communities to foster greater compliance with biosecurity regulations (Hulme et al. 2023). Funding bodies should, therefore, aim to support research that advances broader underpinning issues relevant to more than one sector rather investing solely in taxonomically or sector-focused problems since this will create a greater critical mass of expertise and often result in more effective solutions (Kenna and Berche 2011). Having separate funding bodies for human, animal, plant and ecosystem health is a major barrier to interdisciplinary biosecurity.

Third, with an agreed definition of biosecurity and opportunities to fund interdisciplinary research programmes, the final step is to develop a culture of interdisciplinary thinking in biosecurity. There is considerable opportunity to establish interdisciplinary centres with a focus on biosecurity since it is a subject that is socially relevant and addresses real-world problems that require outputs to support practical actions or interventions. This should encourage researchers to work towards the common good rather than for personal benefits and reputations. Such centres should have a focus on the unity of knowledge, include different disciplines of academic research, involve non-academic participants, such as policy-makers and have a process of continual review to contemplate the broader context of the work (Lawrence et al. 2022). While integration of social science is often promoted as important for interdisciplinary research, the citation analyses indicated that there is already an established community of social scientists working on the socio-political and behavioural aspects of human and animal health. It would seem sensible to encourage them to extend their skillset to both plant and ecosystem health. In addition, the training of the next generation of biosecurity researchers and policy-makers should embrace interdisciplinarity and to do so will require undergraduate and postgraduate education to emphasise the common elements of the biosecurity system, including the similarities in the processes underpinning invasions and outbreaks (Ogden et al. 2019), their societal impacts (Diagne et al. 2021), shared modelling frameworks (Hulme et al. 2020) and management options. This is a quite different philosophy to previous suggestions for training curricula that have emphasised specialisation within a single sector (Minehata et al. 2013; Moritz et al. 2020) or a strong focus on the development of taxonomic skills (Harmon et al. 2022). While knowledge of pathogens, pests and weeds is an essential basis for biosecurity training, greater emphasis on the fundamental conceptual issues underpinning human, animal, plant and ecosystem health is more important for achieving interdisciplinary thinking.

Conclusions

Bibliometric analyses highlight that the field of biosecurity encompasses a wide range of domains from ecology through to economics and requires an interdisciplinary approach to secure human, animal, plant and ecosystem health. Yet, despite a considerable corpus of research addressing biosecurity, the field remains divided by conceptual differences and specialised vocabularies. This situation limits the effectiveness of biosecurity policies and is increasingly being recognised as an obstacle to effective biodiversity conservation, public health and food security. To overcome these constraints requires the building of a global biosecurity community that accepts a broader definition of biosecurity, avoids sectorial jargon and establishes mechanisms to cultivate interdisciplinarity through specialised collaborative centres, cross-sectorial research programmes and conceptually rich training programmes.

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

PEH conceived, analysed, and wrote the paper.

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Data availability

The original data for this research are available through the Clarivate Web of Science.

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Research Article

Biological invasions threaten crops: Alien Himalayan balsam lures and co-opts floral visitors away from cultivated cherry tomatoes

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Abstract

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Copyright: © Kamil Najberek et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International - CC BY 4.0). While it has been demonstrated that invasive alien plants pose a threat to co-occurring wild-growing native plants by attracting their pollinators, we know almost nothing about their impact on

crop pollination. Alien plants with attractive flowers sometimes occur close to crops and may influence yields when they share the same pollinators and have overlapping flowering periods. We present the results of an experiment on the impact of alien balsam (Impatiens glandulifera) on the pollination of cherry tomato (Solanum lycopersicum var. cerasiforme). We verified whether balsam decreases the number of insect visits to the tomato flowers or, conversely, whether balsam may have a positive influence on the pollination rate of the crop. We also assessed crop production with and without the insect visitors. The study was conducted in southern Poland under garden and greenhouse experimental conditions simulating small-scale cultivation of the crop with a neighbouring patch of the alien plant. The studied plants were exposed to insects visiting flowers of the following variants: only one of the two species was exposed or both species were exposed together. Moreover, two factors that may influence insect visits in flowers were assessed: the chemical composition of the floral scent that attracts insects and floral pathogens infesting pollinators that may deter them. The number of insect visits (mainly Bombus pascuorum) on the tomato flowers decreased significantly when the plants were exposed to the alien balsam. Moreover, alien balsam secreted more flower attractants (mainly fatty acids and their esters) than tomatoes, which could explain more frequent insect visits in balsam flowers. However, the floral pathogens probably had a negligible impact on the number of insect visits to the flowers of the two plants. The level of infestation on both studied species was relatively low (I. glandulifera: 5.1% of all pathogen colonies grown in a laboratory, S. lycopersicum var. cerasiforme: 4.2% and 2.6% of all colonies in the garden and greenhouse, respectively) and we found no pathogens known to infect pollinators. It should be noted, however, that some of these pathogens (e.g. Botrytis cinerea, Fusarium oxysporum and Sclerotinia sclerotiorum) are known to cause severe diseases in many crops. Our results revealed that the presence of attractive invasive alien species near small-scale cultivations may negatively affect crop pollination, resulting in smaller fruit size and irregular shape. The impact of such alien species on crop production and the agricultural economy on a large scale requires further study.

Key words: Agriculture, beta-caryophyllene, biofilm organisms, cerise tomato, economy, eradication near cultivations, floral odours, greenhouse

Introduction

Nectar- and pollen-rich flowers offer a reward that many pollinators find hard to resist. As invading alien plants attractive for pollinators grow in progressively larger areas and at higher densities, this effect is significantly enhanced. In Europe, examples of this mechanism include invasions of alien goldenrods (Solidago gigantea and S. canadensis; Moroń et al. (2019)), Japanese knotweed (Reynoutria japonica; Johnson et al. (2019)) and Himalayan balsam (Impatiens glandulifera; Chittka and Schürkens (2001)). All these species are pollination generalists and are thought to play a dominant role in pollinator network structures (Vilà et al. 2009; Vanbergen et al. 2017). They alter pollinator abundance, richness and pollinator community structure (Fiedler et al. 2012), successfully luring and co-opting pollinators in invaded areas. This may result in a decrease in the number of pollinator visits to flowers of co-occurring wild-growing native plants and, consequently, a decrease in their fitness (Chittka and Schürkens 2001; Brown et al. 2002; Morales and Traveset 2009; Albrecht et al. 2014; Herron-Sweet et al. 2016; Goodell and Parker 2017), even to the point of local extinction of the native species (Kleijn and Raemakers 2008).

Invasion of an alien plant may negatively affect native plants by several mechanisms. First, through an increase in the deposition of heterospecific pollen and, consequently, by limiting their reproductive success of the recipient through reducing fruit and seed set (Malecore et al. 2021) and second, through enhancing the transmission of floral primary pathogens that increase the incidence of plant diseases (Najberek et al. 2023). Moreover, large-scale monospecific floral resources formed by invasive alien species might lead to reduced specialist pollinator performance (Filipiak et al. 2017). For example, the pollen of *I. glandulifera* has lower concentrations of proline, an amino acid important for flight metabolism in bumblebees (Drossart et al. 2017).

The opposite scenario, with the positive influence of flower attractive invasive alien plants on native flora and pollinators, is also possible. For instance, if invaders occur at high densities, they may act as "magnet" species that increase pollination of wild plants through, for instance, pollinator spill-over effects (Charlebois and Sargent 2017). This effect may also play a role in crop pollination; however, only a few studies have explored the influence of alien plant species on pollinator–crop interactions (Vanbergen et al. 2017).

Under experimental conditions, Najberek et al. (2021) revealed that invasive alien balsams, *I. glandulifera* and *I. parviflora*, may decrease the pollination of strawberry plants (*Fragaria ×ananassa*). Cunningham-Minnick et al. (2020) revealed that the invasive alien Amur honeysuckle (*Lonicera maackii*) negatively influenced pollinator diversity and pollination services in adjacent corn or soybean cultivations. However, the removal of Amur honeysuckle led to a decrease in the number of large-bodied generalist bees associated with this alien plant. On the other hand, Russo et al. (2016) reported that the invasive alien thistle (*Carduus acanthoides*) did not influence the visitation of pollinators to cultivated plants, such as sunflower (*Helianthus annuus*), honey clover (*Melilotus albus*) or sweet yellow clover (*M. officinalis*). The beneficial influence of alien plant species on crop pollination was also partially demonstrated by Carvalheiro et al. (2011). This study provided evidence that the presence of wild plants (half of which were alien species) within sunflower fields may enhance crop productivity. On the other hand, "pollinator spill-over" from large crop cultivation to adjacent patches of alien plants cannot be excluded. This may enhance the pollination of such species, particularly because many of them develop flowers later in the vegetative season. To date, this trend has been widely demonstrated in studies with crop fields and co-occurring native wild-growing plants (Hanley et al. 2011; González-Varo and Vilà 2017; Trillo et al. 2020; Harris et al. 2023).

We performed a study in southern Poland to test competition for floral visitors between invasive alien Himalayan balsam (*I. glandulifera*; hereafter: alien species) and cultivated cherry tomato (*Solanum lycopersicum* var. *cerasiforme*; hereafter: crop species). Tomato is a buzz-pollinated crop: the pollen is kept locked inside the poricidal anthers and the most efficient way for pollinators to extract it is "buzzing" (Bowers 1975; Banda and Paxton 1991; Franceschinelli et al. 2013). Buzz-pollination behaviour guarantees the release of high volumes of pollen, which is positively correlated with fruit production. Tomato is pollinated mainly by bumblebees (e.g. Teppner (2005)), which exhibit buzz-pollination behaviour (Mesquita-Neto et al. 2018). Bumblebees are also the main pollinators of *I. glandulifera* in the study area (Najberek et al. 2023). Moreover, both plants have overlapping flowering periods (Najberek et al. 2023, https://www.gardenchic.co.uk/) and both commonly occur close to each other in southern Poland (Najberek, pers. observations).

Bumblebees exploit food sources that are as far as 1500 m from their colonies (Osborne et al. 2008). Therefore, within this radius, patches of nectar-rich I. glandulifera (Chittka and Schürkens 2001) around tomato cultivation areas may have a significantly stronger influence upon yields than native wild-growing species that also co-occur with the crop, yet their nectar production is lower. Since pollinator availability determines the quality and volume of tomato production (Velthuis and van Doorn 2006; Franceschinelli et al. 2013; Zhang et al. 2022) and I. glandulifera can affect pollinator availability for strawberry plants (Najberek et al. 2021), we assumed that the co-occurrence of cherry tomatoes with I. glandulifera should decrease the intensity of insect visits to crop flowers and result in lower fruit production. In Poland, the average total area of cultivated tomato fields calculated for June 2010–2021 was ca. 8,000 ha, which accounted for approximately 5% of the total area of cultivated field vegetables in the country (Statistics Poland 2023). It can, therefore, be assumed that the impact exerted by I. glandulifera on crop pollination is meaningful for the country's economy. This effect can be most pronounced in six of the 16 Polish provinces with large productions of tomatoes, including Małopolska (Statistics Poland 2023), where the present study was conducted and where I. glandulifera is particularly widespread (Adamowski et al. 2018).

Pollinators can recognise diseased flowers. It was demonstrated that bumblebees can detect the odour of the parasite *Crithidia bombi* (Fouks and Lattorff 2013) and honey bees were found to avoid flowers infected by the fungus *Ascosphaera apis* (Yousefi and Fouks 2019). When primary pathogens are widely dispersed amongst plant individuals at a particular site, disease-retarding pollination may occur. On the other hand, plants exhibit compensatory mechanisms against pathogen infections, for example, by increased flower production that emit different amounts and/or patterns of attractants (Shykoff and Kaltz 1997; Dötterl et al. 2009). This compensation may increase the reproductive efforts of plants; however, it may also intensify intraspecific and interspecific transmission of harmful pathogens (Durrer and Schmid-Hempel 1994; Shykoff and Kaltz 1997; Adler et al. 2018). To account for the effects of flower infestation and attractants, we identified all floral pathogens,

with an emphasis on pathogens infesting pollinators (e.g. *Apicystis bombi* infesting bees; Vanderplanck et al. (2019)) and we analysed chemical compounds that lure these insects (e.g. linalool, a strong bee attractant; Williams et al. (1981)); *Impatiens glandulifera* differs from cherry tomatoes in the richness of the floral attractants emitted, which may play a role in intensity of insect visits in their flowers.

Our study primarily tested the hypothesis that the presence of invasive alien *I. glandulifera* alongside cherry tomatoes reduces the frequency of insect visits to crop flowers, leading to lower fruit production. Alternatively, we considered that *I. glandulifera* might cause a pollinator spill-over effect, enhancing the pollination and yield of crops in small-scale cultivations. To test these hypotheses, we evaluated the competition for floral visitors between the alien and crop species, along with analysing their floral pathogens and attractants, as these factors could influence insect visitation to flowers.

Methods

Plant species

The variety of tomatoes that we used was cerise, which is a very popular cherry tomato (*Solanum lycopersicum* var. *cerasiforme* (Alef.) Voss) worldwide. The flowers are hypogynous, regular, pendant and typically six-merous, with short calyx tubes and rotated corollas (Cooper 1927). The flowering phase of cerise tomatoes occurs from June to September (mid-flowering: July). They are visited by pollinators (e.g. *B. pascuorum* and *B. terrestris*; Teppner (2005)) mainly for pollen (Franceschinelli et al. 2013). Moreover, this variety is highly disease-resistant (e.g. https://pomidorlandia.pl/en/, https://www.gardenchic.co.uk/); thus, it is successfully cultivated in both garden and greenhouse cultivation in Poland.

Annual *Impatiens glandulifera* Royle mainly occurs along rivers and streams, which allows for its rapid spread (Najberek et al. 2020). However, the species also prefers roadsides, ruderal areas, wetlands, meadows and forest paths (Helmisaari 2010; Helsen et al. 2021). It has one or more pink or reddish (sometimes white) bell-shaped flowers in axillary racemes; the flowers are bilaterally symmetrical and zygomorphic (Clements et al. 2008). The flowering phase of *I. glandulifera* in Poland largely overlaps with the flowering phase of tomatoes, occurring between July and October (mid-flowering: August/September).

The two studied species, *I. glandulifera* and *S. lycopersicum* var. *cerasiforme*, share the same pollinators (Teppner 2005; Najberek et al. 2021, 2023). However, in contrast to tomatoes, *I. glandulifera* attracts pollinators with extraordinarily high volumes of nectar, amounting to 0.3 mg per flower hourly (Chittka and Schürkens 2001).

Cultivation conditions

The experiment was carried out in 2021 under common garden and greenhouse conditions in a cultivation plot at the Institute of Nature Conservation, Polish Academy of Sciences in Cracow (southern Poland). The seedlings of *I. glandulifera* (n = 100) were transplanted in May 2021 from areas near Cracow: Marcyporęba, Zelczyna, Tyniec and Szczyglice (25 seedlings per locality). These plants were cultivated in a garden in pots (1.1 litre capacity) filled with universal garden soil

(pH 5.5–6.5) mixed with sand (a ratio of 3:1). Each plant individual was marked with a unique ID number.

Tomato individuals (n = 122) were germinated in April 2021 from the purchased seeds. The plants were initially cultivated in seedling pots (0.5 litre capacity) under room conditions. On 6 May, the most vigorous plants (n = 80) were transplanted to garden pails (Suppl. material 2: fig. S1) with a 5 litre capacity, which were filled with a standard 100% turf substrate. The plants were marked with unique ID numbers and relocated to a polycarbonate greenhouse (LWH: 405 × 210 × 191 cm). On 8 and 22 July, the substrate was enriched with a biofertiliser.

To avoid frost damage to the cultivated plants, the greenhouse was initially closed at all times and when the day temperature increased, the greenhouse was opened in the morning and closed in the evening. In mid-June, when the day and night temperatures exceeded 16 °C, half of the tomatoes were relocated from the greenhouse to the garden. Since this period, the greenhouse was closed only for the time needed to carry out the tests (see "Ali" treatment in the next subsection) and in the case of weather breakdowns (e.g. windstorms).

The tomatoes were arranged in one patch, -1.5 m away from the patch of *I. glandulifera*. Such an arrangement simulated a common scenario, where crops are directly adjacent to balsam patches. For example, it occurs when farmers cultivate tomatoes for their own food, as well as *I. glandulifera* – either for ornamental purposes or as a food supply for their apiaries (Najberek, pers. observation).

It should also be noted that polycarbonate disturbs the orientation behaviour of insects, which results in their reduced ability to recognise flowers and the environment in greenhouses (e.g. Blacquière et al. (2006)). Although this approach is a serious disadvantage in commercial cultivation, our experimental setup allowed us to almost completely exclude pollinators from the greenhouse. As a result, it was possible to obtain fruits from self-pollinated flowers and compare their quality with fruits from insect-pollinated garden flowers.

We obtained a permission from the Regional Directorate for Environmental Protection in Cracow, which is required for the use of the invasive alien species of European Union concern (No. OP.672.2.2021. KW) and for studying legally-protected bumblebees (No. OP-1.640 1.81.2021.GZ).

Competition for floral visitors and assessment of crop production

Tests of the activity of insects visiting flowers of the two plant species were carried out under warm and windless weather conditions over eight consecutive days between 26 July and 2 August, when the flowering phases of the two species overlapped.

On each experimental day, we assessed whether insects visited *I. glandulifera* more frequently than they visited tomatoes and whether the invasive alien species co-opted the visiting insects from the crop. The studied plants were exposed to three treatment groups, termed "Ali", "Cro" and "AliCro". In the surveys of Ali treatment, only balsams were exposed to insects visiting flowers (simulating a scenario when the balsam is growing alone, not co-occurring with the crop); at that time, all garden individuals of tomato were moved indoors and the greenhouse was closed to isolate tomatoes cultivated inside. In the surveys of Cro treatment, only tomato plants were exposed, both in the garden and in the greenhouse (a scenario when the crop is growing alone in garden or greenhouse); all balsam individuals were moved indoors at that time. In the surveys of AliCro treatment, both balsams

and tomatoes (including tomatoes from the garden and from the greenhouse) were exposed together (a scenario when the two plants co-occur). The data collected during the experiment were used to calculate the number of insect visits recorded per plant individual, per survey and per experimental treatment group.

As the activity of particular groups of insects may be determined by day time (Stelzer and Chittka 2010) as well as by the production of pollen/nectar (e.g. Schmidt et al. (2012)), the sequence in which the treatment groups were exposed was randomly selected for each experimental day. Moreover, a similar number of randomly selected flowering plants (ca. 20 per species) was included on each day; although, for each day, the selected set of individuals was different, some of them were used repeatedly (the excessive individuals were closed indoors). In the garden, the numbers of individuals of each species were adjusted to match the number of tomato individuals flowering in the greenhouse. Moreover, the greenhouse tomatoes were not re-arranged between the subsequent surveys. In turn, the selection and spatial arrangement of particular plant individuals in the garden were modified between the surveys, but not between the study treatment groups (Ali, Cro and AliCro).

The tests started between 8:30 and 11:00 h on each study day and ended between 13:00 and 15:00 h. The survey was conducted by the same researcher and with the same sampling effort of 60 minutes per experimental treatment group, with a 30-minute break before the onset of the survey in the next treatment group. Thus, each study day, the experiment lasted for a total of 180 minutes. In the AliCro treatment, each survey transect (visual inspection) started from the balsam individuals, continued with individuals of tomatoes in the garden and finished with tomatoes in the greenhouse. All flowers of the surveyed individuals were monitored to determine their newly-arriving insect visitors; the duration of each survey transect in the AliCro ranged from approximately 3 minutes (in the case of no visitors) to 9 minutes (when visitors were detected). In the Ali and Cro treatments, the survey transect time was shorter (~2-6 minutes) because some of the plants were closed indoors. The flight of each recorded insect visitor was tracked and the IDs of subsequently visited plants were noted. An insect visit was defined as a single contact of the insect with a flower anther or stigma of a particular plant individual. Since we were not able to assess whether a given visit resulted in pollination, we conservatively did not treat floral visitors as pollinators. However, it can be assumed that the majority of the visiting insects that we recorded were bona fide pollinators. The maximum distance between the flowers and the observer was 1.5 m, which was sufficient to detect any insects. They were identified without disturbing them (93.3% at the species level, 3.9% at the family level and 2.8% at the superfamily level).

Before each survey, flowers on each individual plant were counted (mean n of flowers: *I. glandulifera* = 2.06 ± 1.04 , *S. lycopersicum* var. *cerasiforme* = 2.92 ± 2.51). Air temperature during the survey was monitored using i-Button DS1921G data loggers (with 10-min intervals; Suppl. material 2: fig. S2); wind speed and solar radiation were measured using hand-held environmental meters (Extech 45170CM and SP505). These variables were monitored to include changes in weather conditions that influence pollinator metabolism and activity, for example, an increase or decrease in air temperature during the day (Dixon et al. 2009; Pawlikowski et al. 2020). Notably, the time of day was also included in the analyses because it may impact both the daily patterns of floral resources and the activity of pollinators

(Stelzer and Chittka 2010). During the last survey, the heights of all the plant individuals were measured because taller plants may be more frequently visited by pollinators than shorter plants (Najberek et al. 2021, 2023). In our study, the *I. glandulifera* individuals were taller than the *S. lycopersicum* var. *cerasiforme* individuals (Suppl. material 2: table S1).

In order to assess tomato fruit production involving insect visitors (fruits from the garden) and excluding them (fruits from the greenhouse), size and shape of fruits were measured. Between 27 July and 19 August, 1002 ripe fruits were collected (455 from the garden and 547 from the greenhouse; each of 80 individuals developed fruits; the average number of fruits per single plant was 11.8) and immediately weighed using an analytical balance (Radwag PS 360.R2). Each fruit was classified as healthy (without visible infection symptoms; Suppl. material 2: fig. S3A) or diseased (with major/minor infection symptoms; Suppl. material 2: fig. S4). Diseased fruits with minor disease symptoms were further classified as "saleable" (Suppl. material 2: fig. S4B), whereas fruits with major disease symptoms were further classified as "non-saleable" (Suppl. material 2: fig. S4A). Tomato profiles (n = 856) were photographed against a paper background (Suppl. material 2: fig. S3) and digital images were analysed using ImageJ software (ver. 1.51 k). The fruit area and shape descriptors were calculated (according to Najberek et al. (2020)). The area of one side was assessed for each fruit (Suppl. material 2: table S2), which corresponded to half of the total fruit area. The shape of the tomatoes was assessed using circularity (calculated according to the formula $4\pi \times$ fruit area/ fruit perimeter²) and aspect ratio (the ratio of the major axis of the tomato to its minor axis) data.

Floral chemical attractants

We assumed that the flowers of the two studied plant species differ in terms of the richness of the emitted floral attractants. To investigate this issue, the flowers of *I. glandulifera* (n = 31 from 27 individuals) and *S. lycopersicum* var. *cerasiforme* (n = 87 from 29 individuals) were collected on 26 June and 06 August, respectively. Garden and greenhouse flowers of cherry tomatoes were mixed and analysed without separation because we used this material only for qualitative analysis (Popova et al. 2020; Jakubska-Busse et al. 2022). The samples were placed into 5 ml glass vials filled with 2.5 ml of dichloromethane or hexane (Sigma–Aldrich, 99.9%) at room temperature. These two chemical compounds were used to extract foliar nectar drops. The extracts were stored at -15 °C until they were used for GC/ MS analyses. Samples of the extracts of *I. glandulifera* and *S. lycopersicum* var. *cerasiforme* were prepared separately in dichloromethane and hexane (a total of eight samples of *I. glandulifera* and ten samples of *S. lycopersicum* var. *cerasiforme*).

GC/MS was performed on a GCMS-QP2010SE SHIMADZU gas chromatograph equipped with a mass selective detector (MS scan 17–550 m/z) and a Zebron ZB-5 ms (30 m 0.25 mm; Phenomenex) column. The oven temperature at the start of the measurement was 40 °C and then the temperature was increased at a rate of 4 °C/min until it reached 120 °C; afterwards, the temperature was increased to 320 °C at a rate of 40 °C/min and the oven temperature was kept at 320 °C for 5 min. Helium was used as a carrier gas.

Identification of the extract compounds was carried out using the NIST17 Library. For identification of long-chain hydrocarbons, samples of C16–C42 alkanes were

analysed by GC/MS using the same oven and column parameters and their spectra and retention times were compared with those obtained from the extracts. Qualitative analysis of *I. glandulifera* samples revealed no differences in the composition of the extracts. Similarly, in the case of *S. lycopersicum* var. *cerasiforme*, the qualitative composition of the extracts was comparable amongst all the tested samples.

Floral pathogens

To explore the possible limiting effect of floral pathogens on the pollination of alien or crop species, the flowers of *I. glandulifera* (n = 115) and *S. lycopersicum* var. *cerasiforme* (n garden = 73, n greenhouse = 210) were collected between 30 July and 3 August on the day following each assessment of insect visitor activity, frozen (-18 °C), transported to the mycological laboratory and inserted into a flask (50 ml capacity) filled with 10 ml of distilled sterile water. The content of each flask was shaken (amplitude = 4 cm, n of cycles = 250 for 10 minutes). Subsequently, 1 ml of the washings was placed directly on a Petri dish and filled with Martin medium (BTL Ltd.) at 49.5 °C (Moszczyńska et al. 2011, 2013). After incubating in the dark for 10–14 days at room temperature (21–22 °C), the fungal colonies were counted (Suppl. material 2: fig. S6) and identified using a Leica DM750 microscope and identification keys (Simmons and Ellis 1972; Sutton 1980; Domsch et al. 1982; Ellis and Ellis 1987; Pitt and Hocking 2009; Watanabe 2011). Based on the available literature, the recorded pathogens were classified as harmful or beneficial – both for the insects visiting flowers and for the plants.

Statistical analysis

The data were analysed in R v. 4.0.3 and RStudio v. 1.4.1103 (R Development Core Team 2015). Data collected during the tests of competition for floral visitors (the main and alternative hypotheses testing) was assessed using a generalised linear mixed model (GLMM) fitted using Maximum Likelihood estimation via the template model builder 'TMB' (the glmmTMB package) (Brooks et al. 2017). The number of recorded insect visits calculated per plant individual per survey and per experimental treatment group (n = 1255) was under-dispersed; therefore, the Conway-Maxwell Poisson distribution ('compois') of the target variable was used. The main fixed effects in the base model were the treatment group and plant species status, combined into a single variable (Ali - alien species, AliCro - alien crop species, Cro - crop species; details in the Suppl. material 1). These effects allowed for between-species comparisons for the treatment group AliCro, in which the balsam and the tomato were exposed together. Other fixed effects included in the model were the stem height of individual plants and insect species/group (those of Bombus pascuorum, Apis mellifera and bees from the other groups, as well as syrphids; single flights of *Macroglossum stellatarum* and *B. humilis* were excluded). We also included a weather variable obtained from principal component analysis (PCA), based on air temperature (°C), wind speed (m/s) and solar radiation (w/ m²; Suppl. material 2: table S3). Moreover, two nested random effect structures were included in the model ('1|Survey No./Plant ID/N minutes', '1|Survey No./ Plant ID/N flowers'; Suppl. material 2: table S3). Random effects were applied because of differences between the surveys in terms of: (1) the number of controlled plant individuals per survey, (2) the number of surveys of each controlled plant

individual because some of them were controlled more frequently than others and (3) the number of flowers developed by a plant individual, as it influences the number of insect visits per plant individual. Moreover, the time of each visit was included in the structure as the number of minutes since midnight (e.g. 11:52 a.m. was converted using the following formula: ((11 * 60) + 52) = 712).

The base model was reduced by removing particular fixed effects and the model with the lowest Akaike Information Criterion (AIC) was subsequently chosen (Suppl. material 2: table S3). Within-factor comparisons were conducted using the default R treatment contrasts, while the reference groups in particular models were changed using the "relevel" command.

Tomato production was analysed using three different linear models (for weight, surface area and circularity) and one generalised linear model for gamma distribution (for the aspect ratio). In the model for weight, the data for all the collected fruits (n = 1002) were analysed. Three fixed effects were included in the model: cultivation type (garden/greenhouse), disease symptom occurrence (healthy/diseased) and commercial value of the diseased fruit (saleable/non-saleable; Suppl. material 2: fig. S4); two interactions (cultivation type * disease symptom occurrence, cultivation type * sale possibility of diseased fruits) were also included in the model. In turn, the surface area, aspect ratio and circularity of fruits (n = 856) were analysed using models with a single fixed effect—cultivation type. In the model for circularity, the arcsine transformation of the target variable was carried out.

Besides statistical testing of the hypotheses (main and alternative) and parallel crop production assessment, we conducted the quantitative and qualitative analyses of floral pathogens. For pathogen records, two glmmTMB models were constructed, with the number of colonies per plant per dish used as a target variable (n = 201). As over-dispersion was revealed in these models, a negative binomial distribution was used. In the first model, we compared *I. glandulifera* and *S. lycopersicum* var. *cerasiforme* cultivated in the garden using a model with one fixed effect, namely, the plant species. The nested structure of random effects (1|Plant ID/N flowers/Dish ID) was also included because of differences in the number of flowers placed per dish and in the number of dishes used per plant individual. In the second model (n = 132), *S. lycopersicum* var. *cerasiforme* flowers were compared between the garden and greenhouse cultivation conditions. We used a model with one fixed effect (the cultivation type), two nested random effects (1|Plant ID/N flowers) and one crossed random effect (1|Dish ID; the number of dishes was always three per plant individual).

The composition and richness of the recorded pathogens were calculated using three complementary indices: the Shannon–Wiener, Evenness and inverted Simpson indices (Yin et al. 2019; Twardowski et al. 2022). Three GLMMs with a normal distribution were used to compare the three indices (calculated per plant and per dish; n in each model = 165) between *I. glandulifera* and *S. lycopersicum* var. *cerasiforme* cultivated in the garden. In the model for the Simpson index, the data were logged naturally. As in the models for fungal records, each model had one fixed effect (plant species) and one nested random effect structure (1|Plant ID/N flowers/Dish ID). To compare the indices for *S. lycopersicum* var. *cerasiforme* between the garden and greenhouse cultivation conditions, three GLMMs with a normal distribution were used (n in all models = 98). Each model had one fixed effect (cultivation type), two nested random effects (1|Plant ID/N flowers) and one crossed random effect (1|Dish ID).

Results

Competition for floral visitors and assessment of crop production

A total of 1705 insect visits (*I. glandulifera*, n = 1592; *S. lycopersicum* var. *ceras-iforme*, n = 114; Fig. 1) during 460 insect flights (*I. glandulifera*, n = 416; *S. ly-copersicum* var. *cerasiforme*, n = 44; Fig. 1) were recorded. In both studied plant species, two insect species dominated: the common carder bee *B. pascuorum* and the honey bee *A. mellifera* (Fig. 1). The flowers of *I. glandulifera* were visited 987 times during 131 flights, while the respective values for *S. lycopersicum* var. *cerasiforme* were 91 and 30 (Fig. 1). It should also be stressed that there were two flights of *B. pascuorum* in which both studied plants were visited. The number of flowers visited by *A. mellifera* was lower (n = 593). However, the number of flights was greater (n = 267; Fig. 1). Notably, honey bees visited almost exclusively *I. glandulifera* flowers (99% of visits, 98% of flights; Fig. 1).

Interestingly, while 49% of the individuals of *S. lycopersicum* var. *cerasiforme* exposed during the surveys were visited by insects, in the case of *I. glandulifera*, this value was twice as high, reaching 97% ($\chi^2 = 545.9$, df = 3, p < 2e⁻¹⁶). We revealed that the number of visits to the flowers of *S. lycopersicum* var. *cerasiforme* decreased when this crop was exposed together with the alien species (contrast: estimate = -0.37, SE = 0.03, z = -11.70, p < 2e⁻¹⁶; Fig. 2). The presence of *S. lycopersicum* var. *cerasiforme* had no influence on the insects visiting the flowers of *I. glandulifera* (contrast: estimate = 0.01, SE = 0.01, z = 1.29, p = 0.2; Fig. 2). Therefore, the pollinator spill-over from the crop to the adjacent patch of the alien plants did not occur.



Figure 1. The number of visits by the most common insects visiting flowers of the invasive alien species *Impatiens glandulifera* and cultivated *Solanum lycopersicum* var. *cerasiforme*. The first number in brackets above the bars reflects the number of insect flights to *S. lycopersicum* var. *cerasiforme*, while the second indicates the number of insect flights to *I. glandulifera*. Single flights of *Macroglossum stellatarum* and *Bombus humilis* to *I. glandulifera* are not included in the plot. All insects that had contact with flower anthers or stigmas were accounted for.



Figure 2. Estimated mean number of insects (\pm confidence intervals) recorded from the flowers of invasive alien *Impatiens glandulifera* and cultivated *Solanum lycopersicum* var. *cerasiforme* in the three experimental treatment groups: Ali (alien species exposed), AliCro (both species exposed together) and Cro (crop species exposed). Groups with the same letter above the T-bars are not significantly different.

Considering the numbers of visits per particular insect group (see the numbers in Fig. 1; $\chi^2 = 758.7$, df = 3, p < 2e⁻¹⁶), we revealed statistically significant domination of *B. pascuorum* over syrphids and the other bees (respective contrasts: estimate = -0.46, SE = 0.02, z = -22.74, p < 2e⁻¹⁶; estimate = -0.45, SE = 0.02, z = -18.66, p = 2e⁻¹⁶). *Apis mellifera* also dominated these two insect groups (respective contrasts: estimate = -0.46, SE = 0.02, z = -21.87, p < 2e⁻¹⁶; estimate = -0.45, SE = 0.02, z = -0.45, SE = 0.02, z = -17.61, p = 2e⁻¹⁶). However, there were no differences in the number of visits between *B. pascuorum* and *A. mellifera* (contrast: estimate = 0.0003, SE = 0.002, z = 0.14, p = 0.9) or between syrphids and the other bees (contrast: estimate = 0.01, SE = 0.03, z = 0.37, p = 0.7). Weather conditions had no significant influence on the results ($\chi^2 = 0.06$, df = 1, p = 0.8).

As the polycarbonate effectively prevents insects, we recorded only a single floral visit (of a syrphid species) in the greenhouse, whereas all the remaining insect visit records were from the garden.

Ripe garden tomato fruits were heavier than the fruits from the greenhouse ($\chi^2 = 2129.8$, $F_{1,996} = 129.7$, p < 2.2e⁻¹⁶; Fig. 3). The results for fruit weight and area were concordant (Fig. 3): fruits from the garden also had a greater area than those from the greenhouse (sum Sq = 2207949, $F_{1,854} = 82.55$, p < 2.2e⁻¹⁶; Fig. 3). Moreover, the analysis of the aspect ratio revealed that the garden fruits were more rounded than those from the greenhouse ($\chi^2 = 12.16$, df = 1, p = 0.0005; Fig. 3). The results for circularity showed a similar trend (sum Sq = 0.001, $F_{1,854} = 2.16$, p = 0.1; Fig. 3). There was a significant interaction between cultivation conditions and the commercial value of the diseased fruits: in the garden, there was no difference between weight of the saleable and non-saleable fruits (contrast: estimate = 0.23, SE = 0.64, t = 0.37, p = 0.7; Fig. 4), whereas in the greenhouse, the saleable fruits were heavier than the non-saleable ones (contrast: estimate = 4.69, SE = 1.05, t = 4.48, p = 9.02e⁻⁰⁶; Fig. 4).



Figure 3. Estimated mean values (± confidence intervals) of the weight, surface area, aspect ratio and circularity of *Solanum lycopersicum* var. *cerasiforme* cultivated under garden and greenhouse conditions. The distribution of letters above the T-bars indicates differences between the two cultivation conditions.



Figure 4. Estimated mean values (± confidence intervals) of the weight of *Solanum lycopersicum* var. *cerasiforme* cultivated in the garden and greenhouse conditions between saleable (healthy and slightly diseased; Figs S3A, S4B) and non-saleable (considerably diseased; Fig. S4/A) fruits. The distribution of letters above the T-bars indicates differences in weight between the saleable and non-saleable fruits within particular cultivation conditions.

Floral chemical attractants

There were considerable differences in the compounds composition between the two plant species. The solvent extract of the *S. lycopersicum* var. *cerasiforme* flowers contained various volatile terpenes, for example, α - and β -pinene (Suppl. material 2: table S4). Oxygen-containing compounds were less abundant in the extract. Amongst them, we identified aromatic alcohols, namely, derivatives of benzyl al-
cohols, such as methyl salicylate and eugenol. Additionally, two other compounds, dodecyl acetate and hexadecenoic acid, were present in the extract. The extract also contained various saturated long-chain hydrocarbons. Amongst them, long-chain alkanes, which consist of odd carbon atoms (C25–C31), were the most abundant compounds (Suppl. material 2: table S4).

By analysing the GC–MS traces of *I. glandulifera* extracts, we revealed that terpenes were absent in the samples (Suppl. material 2: table S5). The chemical compounds identified in the extracts included a series of aliphatic saturated alcohols, ketones and aldehydes, for example, hexadecanal and nonadecanol. Amongst oxygen-containing compounds, various fatty acids and their esters, for example, palmitic and linoleic acids, were also detected. The *I. glandulifera* samples contained fewer hydrocarbons than the *S. lycopersicum* var. *cerasiforme* samples. However, in contrast to those of the latter species, 1-pentacosene, 9-nonacosene and squalene hydrocarbons were identified solely in the extract of *I. glandulifera* (Suppl. material 2: table S5).

Floral pathogens

In total, 4541 colonies of 43 taxa (mainly fungal) were identified (Suppl. material 2: table S6; details in the Suppl. material 1). We found no pathogens known to infect pollinators. In turn, primary obligatory pathogens, with invariably negative impacts on plants, accounted for 3.6% of all records. These were Boeremia lycopersici, Botrytis cinerea, Fusarium avenaceum, F. oxysporum, F. sporotrichioides, Phoma herbarum, Rhizoctonia solani, Sclerotinia sclerotiorum and pathogens belonging to the Pythium genus. However, the primary pathogens were rarely detected in the flowers of either of the two species. In the case of *I. glandulifera*, only 5.1% of all colonies were identified as primary pathogens. The values for the garden and greenhouse cherry tomatoes were 4.2% and 2.6%, respectively. Less harmful secondary pathogens, whose presence may even be beneficial for plants, were also present in the analysed flowers (e.g. Alternaria alternata and Cladosporium cladosporioides). We also recorded microorganisms that form biofilms (e.g. Aureobasidium pullulans and bacterial colonies) that protect plants from penetration by primary pathogens. Organisms not associated with the surveyed plants were also detected (e.g. the wood-decaying fungus Dematioscypha dematiicola, probably associated with the wooden construction of the greenhouse basement), as were parasites of fungi (e.g. Trichoderma harzianum; Suppl. material 2: table S6).

The total number of colonies in the garden was more than four times greater for the flowers of *I. glandulifera* than for the flowers of *S. lycopersicum* var. *cerasiforme* (Suppl. material 2: table S6). The dominance of the alien plant species in this respect was confirmed in the GLMM ($\chi^2 = 6.81$, df = 1, p = 0.009; Suppl. material 2: fig. S5A). This result was strongly influenced by the abundant occurrence of a few fungal species and genera (*A. alternata, C. cladosporioides, Cordyceps fumosorosea, D. dematiicola, Penicillium sect. chrysogenum, Phoma herbarum, Ampulliferina* sp., *Basidiomycota* sp., *Exophiala* sp., *Mortierella* sp.) and by the red yeast colony on the flowers of the alien species. Only *T. harzianum* colonies were more abundant in the flowers of *S. lycopersicum* var. *cerasiforme* (Suppl. material 2: table S6).

Amongst the three GLMMs used to compare the composition, richness and diversity of the recorded taxa, models for the Shannon–Wienner and Evenness indices revealed that the taxa detected in *I. glandulifera* occurred significantly more evenly (i.e. without the dominance of a single species) than in the case of

S. lycopersicum var. cerasiforme ($\chi^2 = 13.55$, df = 1, p < 0.001; Fig. 5A; $\chi^2 = 4.10$, df = 1, p = 0.043; Fig. 5B; respectively). Higher values of the two indices reflect more even distributions of pathogen species. In turn, the Simpson index showed that the diversity of taxa was greater in *I. glandulifera* flowers than in *S. lycopersicum* var. cerasiforme flowers ($\chi^2 = 11.03$, df = 1, p = 0.0009; Fig. 5C); a greater number of taxa in the 1/Simpson analysis indicated a lower diversity of pathogens.



Figure 5. Estimated mean values (± confidence intervals) of the three indices assessing the composition, richness (Shannon–Wiener, Evenness) and diversity (1/Simpson) of the microorganisms recorded from the flowers of the invasive alien species *Impatiens glandulifera* and cultivated *Solanum lycopersicum* var. *cerasiforme*. Comparisons between the two plant species (for each species cultivated under garden conditions) are shown in plots **A–C**. In turn, comparisons between *S. lycopersicum* var. *cerasiforme* cultivated in the garden and in the greenhouse are shown in plots **D–F**. The distributions of the letters above the T-bars indicate differences in the indices between the two plant species/cultivation conditions.

A comparison between the greenhouse and garden tomato flowers was also carried out. In the greenhouse, where the air temperature was higher (Suppl. material 2: fig. S2), the total number of colonies was almost eight times greater than that in the garden (Suppl. material 2: table S6). This dominance was confirmed in the GLMM (χ^2 = 5.24, df = 1, p = 0.022; Suppl. material 2: fig. S5B). This result was driven mainly by the abundant occurrence of the following greenhouse taxa: A. alternata, C. cladosporioides, D. dematiicola and Penicillium sect. chrysogenum (Suppl. material 2: table S6). However, abundant colonies of Aspergillus niger, C. herbarum, Penicillium commune, R. solani, S. sclerotiorum, Basidiomycota sp., Exophiala sp. and bacteria also played a role (Suppl. material 2: table S6). In turn, the results obtained for the Shannon-Wiener and Evenness indices were not consistent. The result for the former index was significant ($\chi^2 = 5.04$, df = 1, p = 0.02; Fig. 5D), while for the latter, it was non-significant ($\chi^2 = 0.21$, df = 1, p = 0.6; Fig. 5E), which suggests little relevance of the revealed differences in the composition and richness of the taxa. In turn, the Simpson index results were unambiguous, with greater diversity observed for the greenhouse-grown flowers ($\chi^2 = 3.91$, df = 1, p = 0.048; Fig. 5F).

Discussion

Competition for floral visitors and assessment of crop production

The pollination of crops may be negatively influenced by co-occurring alien plant species. To date, this assumption has only rarely been tested (Carvalheiro et al. 2011; Russo et al. 2016; Cunningham-Minnick et al. 2020; Najberek et al. 2021). In the present study, we verified our hypothesis using invasive alien Himalayan balsam (Impatiens glandulifera) and a cultivated cherry tomato plant (Solanum lycopersicum var. cerasiforme). The results showed that the number of insect visits to flowers of tomato significantly decreased when this crop was exposed together with alien balsam. On the other hand, the presence of the crop had no impact on the number of insects visiting the balsam flowers. Thus, insect spill-over from the crop did not enhance the pollination of the alien species. The lower attraction of the crop to pollinators may be due to the fact that S. lycopersicum var. cerasiforme provides only pollen, whereas I. glandulifera offers not only pollen, but also ample amounts of nectar. The most common pollinator species, Bombus pascuorum, which visits the flowers of the two species, requires both nectar and pollen to survive. Additionally, in tomato flowers, pollen is locked within the anthers and pollinators must use vibrations to release it ("buzz pollination"; Bowers (1975); Banda and Paxton (1991); Franceschinelli et al. (2013)). Therefore, it is likely that collecting nectar from tomato flowers requires more effort compared to alien species, which may influence the flower choice of pollinators when the two species co-occur.

It should be stressed that the present study was carried out under unique experimental conditions for testing the influence of alien plant species on crop pollination (Najberek et al. 2021). A possible limitation of our study design was that we did not capture the entire community of visiting insects, including visitors active before dawn. There was also some variation in the start and end times of the experiments; thus, we did not have strict control over the differences in the daily phenology of pollen maturation and nectar production, which could bias the obtained results. However, we did not aim to provide absolute numbers of visits of the complete array of floral visitors. The aim of this experiment was to assess the relative differences in visitor assemblages and visit intensities between the two studied plant species under controlled conditions. We included time and weather conditions in our analyses and our experimental design allowed us to reduce the possible confounding effects of multiple additional factors (e.g. the size of the alien species patch or crop field area) or a combination of such factors, which may, in turn, strongly affect the results obtained under field conditions. For example, Carvalheiro et al. (2011) carried out such studies under real-field conditions; half of the flowers within crop fields were alien, while the other half were native. The authors indicated that the alien species had a beneficial effect on crop productivity. However, it cannot be unequivocally estimated whether this result was in fact determined by the alien flora, by the native flora or by combined effects. In turn, in our experiment we excluded the possibility of a confounding effect of native plants by exposing the alien species in combination only with the crop. The two species shared the same floral visitors and occurred in similar numbers. Our study mimics a small-scale cultivation. Similar tests under real-field conditions would provide a complete insight on alien plant-crop interactions mediated by pollinators. The inclusion of reference species, for example, wild-growing native plants, in controlled experimental designs would also be valuable for comparing the luring and co-opting potential of native flora of visitors with those of alien species. Such an approach has not been taken thus far.

The presence of pollinators significantly increases the reproductive success of most wild-growing and cultivated plants (Ollerton et al. 2011). Therefore, alien plants luring and co-opting common pollinators may have a negative impact. In agriculture, pollinator deficiency results in economic losses because the yield and quality of many crops are positively correlated with the availability of pollinators (Stein et al. 2017). This dependence was also revealed for S. lycopersicum var. cerasiforme (Velthuis and van Doorn 2006). In our tests, the number of insect visits to tomato flowers in the greenhouse was close to zero; as we expected, visitors did not penetrate into the polycarbonate greenhouse used in the experiment. Polycarbonate protects crops from ultraviolet radiation in that it decreases pest and pathogen pressure (e.g. Kittas et al. (2006)). However, ultraviolet radiation also plays a crucial role in the orientation behaviour of bees and bumblebees. The eyes of bees harbour photoreceptors for ultraviolet, green and blue light; hence, a lack of ultraviolet light results in a reduced ability to recognise flowers and the environment (e.g. Blacquière et al. (2006)). Therefore, it can be presumed that floral resources available outside our greenhouse, namely, tomatoes and balsams cultivated in the garden, were much more accessible floral rewards for insects visiting flowers than were greenhouse resources.

In our study, the garden tomatoes were significantly heavier and had a larger surface area than did the greenhouse fruits. In addition, shape, which is an important commercial characteristic, tended to be more circular in gardens. The only parameter that differed between these two groups was the weight of the saleable fruits. The weight of fruits from the garden was even, while in the greenhouse, the share of very light and very heavy fruits was significant. Therefore, the garden tomatoes obtained in our experiments had greater commercial value, as the price of tomato increases with size and when it has a more round shape (Bai and Lindhout 2007).

Other studies reported that the lack of ultraviolet radiation in polycarbonate greenhouses not only protects crops from pests and pathogens, but also increases their quantity and quality (Kittas et al. 2006). In our study, the result was the

opposite: in polycarbonate greenhouse, with no ultraviolet radiation, the yields were lower than in the garden. Other studies have also revealed a smaller and more heterogeneous fruit size and irregular shape associated with low pollinator activity (Velthuis and van Doorn 2006). It is, therefore, likely that the close-to-zero intensity of visits to the greenhouse tomato flowers resulted in a lower level of pollination by insects.

Importantly, the obtained results are essential in the context of the main problem explored in this study: the possibility of luring and co-opting insects visiting flowers of tomatoes induced by the attractive invasive alien *I. glandulifera*. Co-occurrence with alien species may produce a similar effect of decreasing numbers of pollinators, as was demonstrated in our polycarbonate greenhouse. Additionally, with climate warming, *I. glandulifera* begins flowering increasingly earlier in the season, potentially disrupting crop pollination for a longer duration. For instance, in 2024 in Zakopane (a mountainous region in southern Poland), the first bloom of this species was observed on 20 June (Najberek, personal observation).

Our results indicate that the eradication of *I. glandulifera* with co-occurring tomatoes should be recommended. This approach would not only be beneficial for farmers, but also comply with regulatory frameworks. The European Union law was tightened to limit the impact of several dozens of invasive alien species, including *I. glandulifera* (European Commission 2017). It should also be stressed that tomatoes are valued worldwide (Anwar et al. 2019) and the invasion of *I. glandulifera* has been recorded in countries with the greatest tomato production (e.g. Netherlands, Russia, United States; Anwar et al. (2019); Helsen et al. (2021)). The results of our experiment may, therefore, have economic significance at the global scale.

Floral chemical attractants

The chemical composition of the floral extracts of the two studied species certainly had an impact on the results of the tests of competition for floral visitors. We found considerable differences between the two plant species, suggesting that they use different strategies to chemically attract the same groups of insects (mainly bumblebees and bees) that visit their flowers.

The predominant aroma compound of the *S. lycopersicum* var. *cerasiforme* fragrance was beta-caryophyllene, a strong universal attractant for many groups of insects, including bees. Moreover, we found that the flowers of *S. lycopersicum* var. *cerasiforme* secreted numerous specific attractants, such as docosane, tricosane, pentacosane, hexacosane, heptacosane, octacosane, nonacosane and derivatives. These compounds are long-chain hydrocarbons and are known to attract *B. pascuorum* and *A. mellifera* (Cahlíková et al. 2004; Ferreira-Caliman et al. 2012) — the most abundant tomato and balsam floral visitors in our study. We also detected the presence of dodecyl (lauryl) acetate, which is an attractant for several bumblebee species (Coppée et al. 2008) and methyl salicylate, a floral attractant for Syrphidae (Rodriguez-Saona et al. 2011), which were found to attract these insects to the tomato flowers in this study.

Although we cultivated *I. glandulifera* in pots under garden conditions, it emitted chemical compounds similar to those of *I. glandulifera* in natural habitats (Jakubska-Busse et al. 2023). *Impatiens glandulifera* produces numerous attractants, based on fatty acids and their esters. Amongst them, acids (linoleic, stearic, palmitic, myristic and lauric) and methyl esters (palmitic acid and methyl linolenate) are well-known

attractants for *A. mellifera* and *B. pascuorum* (Le Conte et al. 1990; Cahlíková et al. 2004). Interestingly, in the extracts of *I. glandulifera*, we detected fewer long-chain hydrocarbons (e.g. tricosane, pentacosane, heptacosane and nonacosane) than in the *S. lycopersicum* var. *cerasiforme* extracts. However, some of them (1-pentacosene, 9-nonacosene and squalene) were detected only from the alien balsam. As previous-ly mentioned, long-chain hydrocarbons are well known attractants of *A. mellifera* and *B. pascuorum* (Cahlíková et al. 2004; Ferreira-Caliman et al. 2012). Moreover, alcohols and aldehydes were also identified from *I. glandulifera*. Amongst them, hexadecanal deserves particular attention because it is an attractant for bumblebees (Coppée et al. 2008). Although we did not study all known floral signals that may attract insects (visual, gustatory, tactile or thermal; Raguso (2004); Leonard et al. (2011)), it can be assumed that the attractants secreted by *I. glandulifera* (mainly fatty acids and their esters) are more efficient at attracting insects than the aroma compounds of *S. lycopersicum* var. *cerasiforme* flowers (mainly beta-caryophyllene).

Floral pathogens

We did not find pathogens reported as harmful to pollinators. However, we did record primary pathogens threatening plants. They were more abundant in the flowers of *I. glandulifera* than on tomatoes, albeit the total number of colonies of these pathogens was relatively low (3.6% of all pathogen records in both studied plants). Notably, the low number of primary pathogens could be a result of the abundant co-occurrence of secondary pathogens (e.g. numerous recorded *Alternaria alternata* and *Cladosporium cladosporioides*). These secondary pathogens may even be beneficial to the host plant by reducing infestations by more harmful pathogens (Liggitt et al. 1997). In addition, in our flower attractant analyses, we found that *I. glandulifera* produces two compounds with antifungal activity, 1,4-naphthalenedione 2-hydroxy and 1,4-naphthalenedione 2-methoxy (PubChem 2022). These compounds may be produced in response to infection by the detected pathogens.

Although the recorded primary pathogens probably had only a minor impact on pollinating insects, they may be transmitted to other plant species that share the same pollinators and grow in proximity (Durrer and Schmid-Hempel 1994; Adler et al. 2018). In this study, we identified the primary pathogens of the crops Botrytis cinerea and Fusarium oxysporum. These species are included in the list of the top ten fungal pathogens identified in molecular plant pathology on the basis of their scientific and economic importance (Dean et al. 2012). Botrytis cinerea was found in the flowers of both studied plants, while F. oxysporum was found only in the greenhouse tomato plants. Two other Fusarium species (F. avenaceum and F. sporotrichioides) were also detected in the flowers of I. glandulifera. Fusarium species cause a devastating disease, fusarium head blight (Timmusk et al. 2020). Other primary pathogens that we detected were Boeremia lycopersici and Phoma herbarum. The former was isolated from tomato samples collected in the garden. This pathogen causes leaf spot disease and ascochyta blight in tomato plants (Horst 2013). Phoma herbarum, recorded mainly from I. glandulifera, is known to cause leaf spot disease (Thangaraj et al. 2018). We also detected Rhizoctonia solani and Sclerotinia sclerotiorum in both species. Rhizoctonia solani is one of the most common factors that destroy many plant seedlings (Horst 2013), while Sclerotinia stem rot is one of the most devastating diseases in tomato plants (Mazumdar 2021). Thus, if the recorded primary pathogens are transmitted to other neighbouring plant species,

the progressive invasion of *I. glandulifera* may play a role as a hub in pathogen transmission between plants. Such new hosts may include *S. lycopersicum* var. *ceras-iforme*, as we found that insects visited flowers of both studied species interchangeably. Nevertheless, further studies are needed to confirm our supposition.

We also recorded biofilm organisms and mycoparasitic fungi that increase the resistance of plants to primary pathogens. The former group comprises numerous recorded bacteria, yeasts and fungi (e.g. *Exophiala* species, *Epicoccum nigrum* and *Aureobasidium pullulans*), while the latter includes rarely recorded *Trichoderma* species. Importantly, more than 80% of the biofilm organisms were detected on the flowers of *I. glandulifera*. It can, therefore, be assumed that the flowers of this alien species are more resistant to primary pathogens than those of *S. lycopersicum* var. *cerasiforme* and that this factor contributes to the extraordinary ability of *I. glandulifera* to attract insects visiting flowers and to its invasion ability in general.

Conclusions

We demonstrated the negative influence of the invasive alien species Impatiens glandulifera on the intensity of insect visits to flowers of cherry tomato (Solanum lycopersicum var. cerasiforme), which aligns to our earlier findings on the adverse effects of alien balsam invasions on the pollination of strawberry plants (Fragaria xananassa; Najberek et al. (2021)). In both crop species, the yield and fruit quality increase with the availability of pollinators (Velthuis and van Doorn 2006; Dimou et al. 2008). Therefore, pollinator shortage due to competition with this alien species has a negative impact on crop production. Although we found no pathogen species known to infect pollinators, we recorded pathogens that may pose serious threats to crops (primarily Botrytis cinerea and Fusarium oxysporum). These pathogens may be transmitted between flowers of co-occurring alien and crop plants by visiting insects (Durrer and Schmid-Hempel 1994). The European Union has tightened the law to limit new introductions and spread of I. glandulifera (European Commission 2017). The level of control of this species is still insufficient in Poland. Our study indicates that efforts to control this alien plant species should be intensified near crops which require insect pollinators.

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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

Research idea: KN; study design: KN, AJ-B, KP, IC, WS, MH; surveys and insect identification: KN; microbiological analyses: KP, AK-P; flower attractant analyses: AJ-B, IC, MH; statistical analyses: KN; writing - original draft preparation: KN, AJ-B, IC; writing - review and editing: KN, WS, AJ-B, IC, KP; funding acquisition: KN, AJ-B, IC, KP, WS.

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Data availability

The raw data used to perform the statistical analyses are provided in the Suppl. material 1.

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Supplementary material 1

Raw data used to perform statistical analyses

- Authors: Kamil Najberek, Katarzyna Patejuk, Izabela Czeluśniak, Wojciech Solarz, Marek Hojniak, Agata Kaczmarek-Pieńczewska, Anna Jakubska-Busse
- Data type: xls
- Explanation note: The file includes raw data on recorded floral visitors and fungal pathogens (number of colonies and composition).
- Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.
- Link: https://doi.org/10.3897/neobiota.95.134168.suppl1

Supplementary material 2

Experiment on invasive alien species *Impatiens glandulifera*, cultivated species *Solanum lycopersicum* var. *cerasiforme*, insect visitors of their flowers and floral pathogens

Authors: Kamil Najberek, Katarzyna Patejuk, Izabela Czeluśniak, Wojciech Solarz, Marek Hojniak, Agata Kaczmarek-Pieńczewska, Anna Jakubska-Busse

Data type: pdf

- Explanation note: Supporting tables contain data on stem height of the surveyed plants and data on size/shape of their fruits. In addition, details of model selection in statistical analysis are also provided. Supporting figures include data on tomato cultivation and cherry tomato fruits. Weather conditions recorded during the study are also presented, as well as the culture of fungal pathogens from *Impatiens glandulifera* flowers.
- Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

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Research Article

Comparing the ecological consequences of globally invasive fishes versus their F1 hybrids in recreational fisheries

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Abstract

Recreational angling is a major introduction pathway for non-native fish into freshwaters, where multiple non-native fishes are often released into waterbodies to diversify the angling opportunities. When these non-native fishes are taxonomically similar, then there is concern that their hybridisation will result in F1 generations comprising of novel phenotypes that outperform their parental species, resulting in the impacts of these ecological engineering species being accelerated. Across two water temperatures (18 °C, 26 °C), comparative functional response analyses (CFR) quantified the consumption patterns of the globally invasive freshwater fish common carp *Cyprinus carpio* and goldfish *Carassius auratus*, plus their F1 hybrids, before testing differences in their specific growth rates (SGRs). In CFRs, carp consumed significantly more prey at 18 °C than the other fishes, and with no differences between any of the fishes at 26 °C. SGRs also did not differ substantially between the fishes at either temperature. These results suggest that hybridisation between the high impacting parental species did not produce novel phenotypes of high ecological performance that could accelerate their ecological impacts in invaded ecosystems. Accordingly, the ecological risks of their use in recreational angling remain an issue that is primarily associated with the parent populations, and this can be reflected in their invasion management.

Key words: Common carp, comparative functional response, goldfish, heterosis

Introduction

Recreational angling remains an important introduction pathway for non-native freshwater fishes, where the aims of introductions include diversification of target species and to increase angler satisfaction with their catch-related experiences (Carpio et al. 2019; Hirsch et al. 2021). However, these introduced fishes often develop invasive populations, with biological invasions recognised as a major driver of biodiversity loss (Britton 2023). Given that fishes released for freshwater angling enhancement are generally large-bodied and of high trophic position then their invasive populations can have strong top-down effects that disrupt ecosystem functioning (Britton 2023).

Introductions of freshwater fishes for angling have resulted in a relatively small number of non-native fishes developing invasive populations globally, such as the North American largemouth bass *Micropterus salmoides* (Pereira and Vitule 2019). Ecological concerns on their invasive populations relate to the interactions with native prey fishes, where their increased predation pressure can lead to substantial



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Copyright: [©] Ali Serhan Tarkan et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). declines in prey population abundances (Gratwicke and Marshall 2001). Omnivorous species such as common carp *Cyprinus carpio* and goldfish *Carassius auratus* have been used to enhance recreational fisheries across much of Europe, with releases of both species being commonplace in many waters, despite high ecological concerns through middle-out effects (Britton et al. 2010).

Where introductions of multiple non-native species are released into novel communities that are taxonomically similar, such as carp and goldfish, then this raises additional concerns over their hybridisation, as this can alter the functional traits and ecological interactions of the hybridised progeny versus their parental species (Selz and Seehausen 2019). This results from the generation of novel phenotypes arising from the combining of alleles that have not segregated before in the same population (Selz and Seehausen 2019). Although hybridisation results in a general surge of genetic variation from the admixed divergent genomes and the acquisition of specific adaptive traits through lateral gene transfer and introgression (Seehausen 2004), its effects on ecological performance are unpredictable. For example, providing that the interactions between the alleles do not result in intrinsic incompatibilities then transgressive segregation can result in the hybrids outperforming parental taxa via adaptive diversification from the novel combination of parental traits and/ or expression of new traits (Kagawa and Takimoto 2018; Nieto Feliner et al. 2020). Conversely, introgression can result in the performance and fitness of hybrids being lower than their parental species through outbreeding depression (Pregler et al. 2023). In hybrids of the F1 generation, the general patterns tend to be an expression of high hybrid vigour through heterosis, where their expressed traits are superior in performance to those of their parents (Šimková et al. 2021; Dong et al. 2022). This is evident in the ecological impact observed in North America, where hybrids of the native Pecos pupfish (Cyprinodon pecosensis) and the invasive sheephead minnow (Cyprinodon variegatus) are ecologically superior to their parent species through higher growth rates and greater swimming endurance (Kodric-Brown and Rosenfield 2004). Additionally, research on hybrid forms of endemic and invasive Barbus spp. in European freshwater systems revealed these hybrids had larger body lengths for their age than pure endemic forms (De Santis et al. 2021). Moreover, the Barbus population with the largest trophic niche, had the highest number of introgressed alleles with invasive European barbel (De Santis et al. 2021).

Heterosis in the F1 generation is especially important to consider when the parental species are both high impacting non-native species of global concern, such as in carp and goldfish. Both species prefer waters of > 20 °C and are highly invasive globally with foraging behaviours that drive dietary overlaps with native fishes and strongly modify ecosystem functioning (Britton et al. 2010; Britton 2023). In recreational freshwater fisheries where these fishes co-exist, fertile hybrids are often produced (Hänfling et al. 2005). In the England and Wales, hybrids between these species are produced in hatcheries and are then frequently released into lentic catch-and-release fisheries to enhance angling performance. With England and Wales having a temperate climate with water temperatures only occasionally exceeding 20 °C (Amat Trigo et al. 2024) then the ecological performance of these fishes might be constrained in current conditions but accelerate as waters warm through climate change (Ruiz Navarro et al. 2016). Accordingly, testing the performance of these fishes across a temperature range enables their performances to be tested in varying thermal conditions that are ecologically relevant (Ferreira et al. 2014). Given that temperature is a key driver of metabolic rates in ectotherms, influencing their growth rates, feeding behaviour, and overall ecological performance, it is crucial to examine how these species and their hybrids respond to temperatures that reflect both their thermal optima and potential environmental variations (Britton et al. 2010), especially with rising global temperatures altering the thermal landscape of aquatic ecosystems (Priya et al. 2023).

Comparative functional responses (CFR) and specific growth rates (SGR) can be used as proxies for ecological performance of invasive species and applied to forecasting the ecological effects of existing, emerging, and future invasive alien species (Dick et al. 2017; Britton et al. 2018). The functional response specifically examines the relationship between resource availability and consumer uptake (Holling 1959) and can, for example, estimate maximum consumption rates. This approach has proven particularly useful in deriving per capita impacts of invasive non-native species when used comparatively with trophically analogous native species (e.g., Alexander et al. 2014; Dickey et al. 2018). Specific growth rates (SGRs) can test the effect on body mass of how co-existing species compete for fixed food resources versus the species in allopatry, helping identify which species are the superior competitors and the effect of their resource acquisition at individual and group level (Britton 2018). Consequently, CFRs and SGRs provide highly complementary tools for assessing the outcomes of novel interactions of invasive fishes.

The aim here was thus to provide the evidence base for the risk analysis of hybrids arising from the introgression of genes between taxonomically similar species using carp and goldfish as model species to experimentally test their foraging behaviours (as CFRs) and growth performance (as SGRs) versus their first-generation (F1) hybrids in contexts of two contrasting temperatures. We predict that the ecological performance of the F1 fish will be superior to both non-hybrid carp and goldfish through higher consumption rates that provide superior competitive abilities when in sympatry with their parental species which results in faster growth rates, with this heterosis being independent of temperature.

Materials and methods

Experimental fish

The experimental carp, goldfish and F1 hybrids were from the same hatchery in Southern England, where the fish were produced from the same parental lines, and with experimental fish exposed to the identical rearing conditions. Both parental species have thermal optima > 20 °C and critical thermal maxima > 30 °C (Britton et al. 2010; Ferreira et al. 2014). All fish were age 0+ years and of initial body mass 2.0 to 6.0 g on arrival in the laboratory, where they were then individually tagged (7 mm passive integrated transponder tag), and acclimated for 20 days (18 °C; 16:8 h light: dark cycle). Outside of experiments, the fish were held in stock tanks (90 L, flow-through system; n = ~30 fish per tank, species held separately) and fed a maintenance diet of crushed pelletized fishmeal. As the initial CFR experiments were completed at 18 °C, the fish were initially held in their stock tanks at this temperature.

Comparative functional response experiments

For CFR experiments, individual fish were exposed to Chironomid larvae as prey resources in 10 L tanks at 18 °C following a 24-hour starvation period and a 4-hour acclimation period to their experimental tank. Food densities were 4, 8, 16, 32 and 64 larvae (and 128 for the 26 °C experiment). Food exposure was for

one hour, after which the number of larvae consumed was quantified, with three replicates per prey density per species. When all replicates were completed, the fish were returned to the stock tanks. The water temperatures of both the stock and experiment tanks were then increased to 26 °C over 8 days and, following a 5-day acclimation period, the CFR experimental process was repeated. With the fish being PIT tagged, no individual fish was used more than twice in CFRs, with a minimum of five days between use (with fish used randomly across the experiments).

Values of the CFR parameters attack rate (*a*) and handling time (*h*) were calculated for each species and temperature using maximum likelihood estimation (MLE) in the Random Predator Equation (Rogers 1972), completed in the R package '*Frair*' (Pritchard et al. 2017). The equation assumes a Type II functional response and the non-replacement of prey, where $N_e = N_0 (1 - \exp(a(N_e h - T))))$, with $N_e =$ number of prey eaten, $N_0 =$ initial density of prey, *a* = attack rate, *h* = handling time and *T* = total time. Analyses also provided the significance of differences in *a* and *h* between the species (Pritchard et al. 2017; Cuthbert et al. 2019). To visualise uncertainty, 2000 non-parametric bootstraps enabled empirical 95% confidence intervals to be fitted around the functional responses, which were used to provide CFR plots between the parental species/ F1s, and water temperature.

Specific growth rate experiments

Following completion of CFRs, all water temperatures were returned to 18 °C and the fish acclimated for 10 days in the stock tanks. The competitive growth performance of the fishes was then tested using SGRs through completion of co-habitation experiments completed in tanks of 25 L. Experimental treatments used controls (species/hybrid in allopatry; n = 6) and treatments (combinations of two species in sympatry; n = 3 + 3; and all species in sympatry; n = 2 + 2 + 2), each replicated three times. Each species per experimental treatment was batch weighed (to 0.01 g) before being released into their tanks, where they were held at 18 °C and fed a daily food ration (crushed pelletised fishmeal) at a mean of 2% starting body mass. After 15 days, the fish were removed from their tanks, re-weighed, returned to their tanks and the water temperature increased to 26 °C over five days before the experimental process was repeated. For each species/hybrid, control and treatment, and water temperature, SGR was determined from $([(lnW_{t+1}) - lnW_t)/J/n) \times 100$ (Equation 1), where W_t = total starting weight of the species in the tank, W_{t+1} = total finishing weight, n = number of fish, and t_{t} = number of days between W_{t} and W_{t+1} . A generalised linear model (GLM) tested the differences in SGR between treatments for each species, where SGR was the dependent variable, treatment was the independent variable, and total starting mass of fish per replicate used as an initial covariate and retained in final models when its effect was significant. Model outputs were the overall significance of the model and the mean SGR values (± 95% CI) according to species and treatment. All analyses were performed in R (version 4.2.3; R Core Team 2023).

Results

Comparative functional responses

The functional responses of all species at all temperatures were Type II and significant (Fig. 1; Suppl. material 1). The 95% confidence intervals of consumption

rates at 18 °C suggested carp had significantly higher rates than goldfish and hybrids, which had similar values to each other (Fig. 1). All species revealed higher consumption rates at 26 °C versus 18 °C, with no significant differences between them. Attack rates of carp and hybrids were also significantly higher at 26 °C versus 18 °C, but not for goldfish. For all species and the hybrids, handling times were lower at 26 °C than 18 °C (Table 1; Suppl. material 1). There were also significant differences in these metrics between the species at both temperatures, with the attack rate of carp being lower than the other fishes at both temperatures (Table 1).

Specific growth rates

Increased fish mass occurred in all SGR treatments at both temperatures (Fig. 2; Suppl. material 1). The effect of treatment on SGR was significant for each species (GLM: carp: Wald $\chi^2 = 452.39$, df = 22, p < 0.0001; goldfish: Wald $\chi^2 = 130.91$, df = 23, p < 0.001; hybrid: Wald $\chi^2 = 128.92$, df = 22, p = 0.002), where starting mass as a covariate was significant for carp (p = 0.01) and hybrids (p = 0.03) (retained in



Figure 1. Comparative functional response curves for carp, goldfish and their F1 hybrids at 18 °C (top plot) and 26 °C (bottom plot). Shaded areas around the curves represent 95% confidence intervals generated by boot-strapping. Note differences in values on both axes between the plots. The mean prey consumed was over the period of 1 hour.

Table 1. First order linear coefficient results from logistic regressions for the predator and prey combinations (A). All values indicate a Type II functional response. Parameters of the comparative functional responses, with statistically significant differences in the parameters between species ($\alpha = 0.05$) in bold. *a* = attack rate, *h* = handling time (B). *Z* and *P* values are statistical outputs from regression that indicate whether *a* and *h* differ significantly between the comparator species.

(A)				
Temperature	Species	Linear coefficient	Р	Pseudo R ²
18 °C	F1	-0.09	< 0.0001	0.63
-	Goldfish	-0.06	< 0.0001	0.67
-	Carp	-0.03	0.0001	0.65
26°C	F1	-0.06	0.001	0.69
	Goldfish	-0.04	< 0.0001	0.70
	Carp	-0.02	< 0.0001	0.68
(B)				
18°C	F1/Goldfish	F1/Carp	Goldfish/Carp	
a	12.72/7.67	12.72/3.43	7.67/3.43	
Ζ	-1.15	2.34	2.11	
Р	0.25	0.02	0.04	
h	0.04/0.05	0.04/0.01	0.05/0.01	
Ζ	1.10	8.11	8.77	
Р	0.27	< 0.001	< 0.001	
Pseudo R ²	0.63/0.67	0.63/0.65	0.67/0.65	
26°C				
a	42.18/6.89	42.18/3.46	6.89/3.46	
Ζ	-8.55e7	-19.59e7	3.28	
Р	< 0.001	< 0.001	0.001	
h	0.010/0.008	0.010/0.008	0.008/0.008	
Ζ	-3.39	-12.54	-0.19	
Р	< 0.001	< 0.001	0.87	
Pseudo R ²	0.69/0.70	0.69/0.68	0.70/0.68	

final models), but not goldfish (p = 0.16; removed from final model). At both temperatures and all species, SGRs in the allopatric controls were generally lower than in the sympatric treatments, but with the effect of temperature on SGR being minor; where elevated SGRs were apparent then this was at 18 °C rather than 26 °C (Fig. 2).

Discussion

There was no evidence to suggest heterosis was apparent in the performance of the F1 hybrids across both experiments, with their performance in CFRs being weak versus carp at 18 °C and differences in consumption rates not being significant at 26 °C. Their CFR metrics were significantly higher at the elevated water temperature, but this was also apparent in goldfish, with the maximum consumption rates of all of the fishes being similar at this elevated temperature. The SGR experiment also did not indicate any substantially enhanced performance in the F1 fish versus the other fishes, nor was there a strong effect of temperature on SGR, most likely due to the feed rations being maintained at a constant level across both temperatures.

Heterosis is a common outcome of hybridisation in early generations, as observed in invasive plants (Hahn and Rieseberg 2017), where admixture can increase performance across multiple generations (Li et al. 2018), and in fishes, where similar patterns of heterosis were detected in crosses of three-spine stickleback *Gasterosteus aculeatus* from different lake populations (Thompson and Schluter 2022).



Figure 2. Mean specific growth rates of cohabitation experiments for carp ('Ca'), goldfish ('GF'), and their F1 hybrids ('F1') at 18 °C (upper) and 26 °C (lower). * denotes significant differences between treatments.

Interspecific hybridisation is common in freshwater fishes (Bolnick 2009), with the few performance studies on inter-specific hybridisation involving invasive fishes in the wild yielding equivocal results. For example, low larval mortality in the F1 generation of non-native red shiner *Cyprinella lutrensis* and native blacktail shiner *Cyprinella venusta stigmatura* was suggested as evidence of heterosis (Blum et al. 2010). However, in early generation hybrids of Asian bighead carp species (*Hypophthalmichthys* spp.), nutritional performance was intermediate between the parental lines, with advanced generations then becoming increasingly similar to parental species (Liss et al. 2016). However, hybrids of *Barbus* spp. were larger and had larger trophic niches than endemic parent species (De Santis et al. 2021). Heterosis in the performance of F1 generations of fishes mixed in aquaculture is more evident (Šimková et al. 2022) where advantages can include decreased viral susceptibility and faster growth rates (Bryden et al. 2004).

Our results, generated using hybrids and parental species under controlled conditions, represent novel outcomes as we could find no similar studies comparing the ecological performance of such high-impact invasive species versus their F1 generation. Although we concluded that F1 hybrids were not more impactful than their parental species, this should not be interpreted as an indication that they pose no management concern. On the contrary, our findings suggest that F1 hybrids are equally impactful, which implies that they should be managed with the same level of concern as the parental species. Furthermore, while only F1 hybrids were tested in this study, it is important to recognise that advanced generations of hybrids can exhibit increased similarities to the parental species, as seen in other studies (Hänfling et al. 2005; Liss et al. 2016). This highlights the necessity of considering the potential ecological impacts of these advanced generations in future research and management strategies. Therefore, our study provides critical insights into the ecological performance of F1 hybrids, but with further investigation needed into the long-term ecological impacts of hybridisation, where there is a need to better understand the behaviour of later-generation hybrids.

This absence of transgressive segregation and heterosis in the hybrids of these globally invasive fishes is then important for their risk screening within management frameworks regulating the release of non-native fishes in recreational fisheries (Vilizzi et al. 2019; Copp et al. 2021). Both parental species have been assessed in risk screening as being of high ecological risk where, for example, they were assessed as representing the highest ecological risk of all non-native fishes introduced into freshwaters in England (Britton et al. 2010). Consequently, there was high concern that their hybrids would be composed of novel phenotypes that result from combinations of alleles that were previously always segregated (Selz and Seehausen 2019). Given there was no evidence that the F1 generation had superior performance in their foraging and growth rate, especially at 18 °C where carp were superior, but also at 26 °C where differences between the species were generally not significant, then this represents an important outcome for their scientifically informed management. Accordingly, the ecological concerns with introductions of these species remain with their parental populations and so where risk-based invasion management programmes are implemented to minimise the impact and dispersal of these species, they do not need to account for the possibility of these species producing hybrids that will accelerate their ecological impacts. This means that where invasive populations develop from fishes initially introduced for recreational angling then control and containment programmes could be effective in reducing the impacts of their populations (Britton et al. 2023), with the evidence base for such management decisions enhanced by the results presented here.

These results are particularly relevant in the context of climate change, which is expected to increase water temperatures globally (Britton et al. 2010; Ruiz Navarro et al. 2016). The lack of significant differences in performance between the hybrids and parental species at 26 °C suggests that, under warmer conditions, the hybrids do not exhibit superior ecological performance. This is an important consideration as rising temperatures might otherwise be expected to exacerbate the impacts of invasive species (Fobert et al. 2013), including their hybrid forms. The similar performance across species at elevated temperatures indicates that while these fishes can thrive in warmer conditions, the hybrids do not pose an increased threat beyond that already posed by the parental species. This finding contributes to our understanding of how these species might behave under future climate scenarios, where water temperatures in many regions may exceed the current norms (Emiroğlu et al. 2023). Furthermore, the co-habitation treatments in this study allowed for an assessment of how these species interact when placed in shared environments. The absence of significant differences in performance between the hybrids and their parental species during co-habitation suggests that the hybrids do not possess competitive advantages that would allow them to outcompete their parental species in shared habitats (but see Kodric-Brown and Rosenfield 2004; De Santis et al. 2021). This finding is important for understanding potential interactions in the wild, where these species might co-occur. However, it is important to note that while these findings are significant for the species studied (carp and goldfish), they may not necessarily generalise to other fish species. The specific ecological and genetic contexts of different species combinations could lead to different outcomes, and caution should be exercised before applying these results to other cases.

It is also important to acknowledge the limitations of this study. One limitation is that only the F1 generation was examined, and the long-term ecological impacts of advanced generations of hybrids were not assessed. As other studies have shown, advanced generations of hybrids can exhibit increased similarities to parental species, potentially leading to different ecological outcomes (Liss et al. 2016). Additionally, the controlled conditions of the experiments may not fully replicate the complexities of natural ecosystems, where factors such as predation, competition, and varying resource availability could influence the performance of these species and their hybrids.

Future research should focus on evaluating the performance of advanced hybrid generations under a wider range of environmental conditions, including varying temperatures and more complex ecological interactions. It would also be valuable to investigate the potential for hybrid vigour or outbreeding depression in these later generations, which could have significant implications for their ecological impact. Moreover, field studies that examine the behaviour and performance of hybrids and parental species in natural settings would help to validate and extend the findings from this controlled study.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

Author contributions

JRB conceived the study and experimental design. AST and IK collected data. All authors analysed data, and drafted and edited the manuscript, and agree to be held accountable for the work performed therein.

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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

Raw data for functional response and specific growth rate

Authors: Ali Serhan Tarkan, Irmak Kurtul, J. Robert Britton

Data type: xlsx

Explanation note: All raw data collected through experiments in the study.

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Research Article

Reliable molecular detection of small hive beetles

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NeoBiota

Abstract

Invasive species require adequate reliable detection methods to mitigate their further spread and impact. However, the reliability of molecular detection methods is often hampered by both false positives (Error type I) and false negatives (Error type II). At present, the reliability of the four published molecular detection methods for small hive beetles (SHB), *Aethina tumida*, has not been rigorously evaluated considering their extensive genetic diversity. Here, we performed intra- and interlaboratory comparisons of the four available methods using SHB samples representing 78 regions from 27 countries on five continents, beetles from the same genus (*Aethina concolor, A. inconspicua, A. flavicollis* and *A. major*), as well as western honey bees, *Apis mellifera*, and ectoparasitic mites *Varroa destructor*. The data show that the Idrissou et al. (2018) and Li et al. (2018) methods avoid both false positives and false negatives probably due to lower sensitivity to nucleotide mismatches on the primer and probe's target sequences. Further, the Li et al. (2018) method can be considered more sensitive because the fluorescent amplification curve crosses the threshold at lower Cq values compared to the Idrissou et al. (2018) one. In light of our data, the Li et al. (2018) method is the most reliable molecular diagnostic tool for SHB. We therefore recommend using this method as it will contribute to management efforts of this invasive species.

Key words: Aethina tumida, inter-laboratory comparison, qPCR, ring test

Introduction

The small hive beetle (SHB), *Aethina tumida*, is a parasite and scavenger of honey bee colonies that is continuing to invade the world since it was first noticed outside its natural distribution, in Africa, south of the Sahara, in 1996 in the USA (Hood 2000). As infestation of honey bee colonies with *A. tumida* did cause severe damage to apiculture in all the new areas where *A. tumida* has been introduced to (Neumann and Elzen 2004; Ellis and Hepburn 2006), it has been added to the lists of notifiable diseases of the World Organization for Animal Health (WOAH) and the European Union (EU). However, despite comprehensive elimination and contingency efforts, it already has established local populations on every continent except Antarctica and it is likely to continue spreading (Neumann et al. 2016; Schäfer et al. 2019).

A reliable method for the early detection of SHB specimens in places where they are not endemic provides the opportunity to have a cost-effective management of the situation which will look to prevent the initial establishment of SHB, and therefore, minimize the ecological and economical effects of this invasive species. However,



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Copyright: © Orlando Yañez et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0). despite this obvious advantage, the reliability of the different DNA-based detection methods for SHB is unknown. The molecular methods based on the detection of SHB's DNA have the advantage of identifying not just the adults but the insect's early developmental stages as well. Indeed, the taxonomical identification of early stages as eggs is a difficult task if it is based only on the morphology. However, a potential limitation of most molecular methods for the detection of SHB is that they were designed with limited information of SHB's DNA variability. Actually, the primers (and probes) from most methods were designed with DNA information from specimens collected in introduced areas and few specimens from the African continent where the beetle is widely distributed and where the higher genetic diversity is expected. Therefore, it is quite important to test the reliability of the molecular methods using a much larger number of SHB specimens from their continent of origin (Idrissou et al. 2019).

Over the last 15 years several PCR methods had been developed for the detection of SHB. This study compares four genetic detection methods to evaluate their effectiveness, sensitivity and specificity, identifying the strengths and limitations of each method, aiming to identify the most accurate one. Three of those diagnostic methods were designed for using the hydrolysis probe technique (Ward et al. 2007; Li et al. 2018; Silacci et al. 2018). Those assays include a sequence-specific, oligonucleotide probe labelled with a fluorescent reporter and a quencher of fluorescence at opposite ends, in addition to the sequence-specific PCR primers. The hydrolysis method exploits the 5' to 3' exonuclease activity of the Taq polymerase. At the PCR extension step, once the polymerase reaches the probe, its exonuclease activity degrades the probe cleaving off the fluorescent reporter. As a result, it is separated from the quencher, resulting in a fluorescence signal. Probe-based qPCR enables the amplification of more than one target in a single reaction using different reporters with distinct fluorescent spectra. As this technique uses specific primers and probes to the target sequences, it is regarded as a technique with very high specificity. Besides the methods using the hydrolysis probe technique, also a detection method designed for conventional PCR (Idrissou et al. 2018) was evaluated. However, for this study the method was modified to test if those primers were suitable for the SYBR Green qPCR method. The qPCR non-specific detection method uses SYBR Green as fluorescent dye. This dye emits fluorescence when binds to double stranded DNA (dsDNA). Therefore, the fluorescence intensity is proportional with the concentration of dsDNA. It is considered a non-specific method because the dye binds to dsDNA, independent of the nucleotide sequence. The absent of specific-sequence fluorochrome-labelled probe make its use less expensive. However, the specificity relied entirely on the design of the primers to avoid the risk of nonspecific PCR amplifications. Commonly, this is verified assessing the melting temperature (Tm) of the amplicon by melting curve analyses that take place after the qPCR runs.

There are two error types that are of special importance to evaluate, error types I and II. Error type I, also known as a false positive, occurs when a method incorrectly identifies the presence of SHBs when they are absent. Error type II, also known as a false negative, occurs when a method fails to detect the presence of SHBs when they are actually present. For the evaluation of those parameters of the SHB qPCR detection methods, it was particularly necessary to test them using an extended collection of SHB specimens. In this study, the collection has representative specimens from the three known SHB phylogenetically clades (Liu et al. 2021) and were sampled from 4 non-endemic continents and a significant contribution from Africa (44 out of 78 total regions, and 16 out of 27 total countries) since it is the continent of the species origin holding the major genetic diversity (Idrissou et al. 2019). This is important to highlight as most of that genetic information was not available when the tested detection methods were developed, which implies that some haplotypes may have not been considered during the design of these methods, which may have consequences in their accuracy.

Finally, for further validation of the detection method comparison, we performed a blind ring test and an inter-laboratory comparison test between laboratory partners dedicated to the detection of SHB. For the ring test, the participating laboratories blindly tested selected SHB haplotypes using their own routine methods. For the inter-laboratory comparison test all participating laboratories used the selected most accurate method to tests its reproducibility and sensitivity across these laboratories.

Materials and methods

Comparison of methods

The objective of these tests was to establish the capabilities of different proposed qPCR methods to reliably confirm the detection of SHB.

Samples

Adult SHB (N = 83) representing 78 regions from 27 countries on five continents from the collection at the Institute of Bee Health (IBH, University of Bern, Switzerland) were selected (Suppl. material 1: table S1). Beetles from the same genus (*Aethina concolor, A. inconspicua, A. flavicollis* and *A. major*; N = 1 each; Suppl. material 1: table S1) were also selected and used to test the specificity of the methods. Workers of western honey bees *Apis mellifera* (N = 2) and ectoparasitic mites *Varroa destructor* (N = 2) collected in Switzerland were also included. After collection, all beetle samples were preserved in 70% ethanol, transported at room temperature and stored at -80 °C. The DNA extraction (from the whole specimen bodies), DNA yield and purity (using a spectrophotometer) and the Cytochrome Oxidase I gene (COI) barcoding protocols are described in Idrissou et al. (2019).

Selection of methods

Four DNA-based published methods were considered (Ward et al. 2007; Idrissou et al. 2018; Silacci et al. 2018 and Li et al. 2018). For internal control, a hydrolysis probe assay targeting a common region of the 18S rRNA gene was used (Silacci et al. 2018). The sets of primers and probes are detailed in Suppl. material 1: table S2. At the IBH, all the specimens detailed in the "Samples" section were tested following the protocols as described by Ward et al. (2007); Silacci et al. (2018) and Li et al. (2018), see Suppl. material 1: table S3. In contrast, the PCR conditions described by Idrissou et al. (2018) were adapted to SYBR Green qPCR conditions (Suppl. material 1: table S3).

Ring test using routine detection methods

The objective of the ring test was to establish the proficiency of the participant laboratory's routine method for the detection of SHB.

SHB DNA from single specimens among the three major SHB phylogenetic clades (clade A: Italy (Cosenza-Calabria), clade B: Burkina Faso (Bobo Dioulasso) and Tanzania (Arusha), clade C: Philippines (Davao); Liu et al. 2021) were tested blindly by the four participant laboratories. Besides belonging to a particular phylogenetic clade, those samples were selected because their detection status was not uniform among the methods described above. In addition, to test for interspecific cross detection, DNA samples from single individuals from *A. concolor* (Australia), *A. flavicollis* (South Korea) and *A. mellifera* (Switzerland) were also included in this assay (see "Samples" section). Three technical replicates per sample were provided in 20 μ l volume per replication. DNA samples were prepared at the IBH and delivered to the other three laboratories (Table 1) on dry ice for preservation.

The routine method used by each laboratory and the respective amplification conditions are described in Suppl. material 1: table S4.

Inter-laboratory comparison using the selected detection method

The objective was to establish the proficiency of the Li et al. (2018) SHB probebased qPCR detection method under laboratory conditions of each participant.

The DNA samples (including the replicates) used in the ring test assay were used as well for the inter-laboratory comparison. In addition, each laboratory was also provided with ten-fold serial SHB DNA dilutions (from $5*10^{-3}$ to $5*10^{-9}$ ng/µl) in order to determine the sensitivity of their qPCR assays for this method. The SHB DNA used for the dilutions belong to a sample from Clade B (Burkina Faso, Bobo-Dioulasso), which was previously shown to be positively detected by the described Li et al. (2018) method. Each dilution was provided with three technical replicates (20 µl volume each). The primers and probes for the COI (Li et al. 2018) and 18S rRNA (Silacci et al. 2018) regions were also provided to each laboratory by the IBH. Both sets of primers and probes were set to work simultaneously in multiplex. The qPCR conditions were performed following Li et al. (2018) (Suppl. material 1: table S3).

Table 1. Ring test for the comparison of SHB PCR detection methods. Specimens of *Aethina tumida*, *Aethina flavicollis*, *Aethina concolor* and *Apis mellifera* were screened (blind test) by each participating laboratory using their own routine detection method. Positive detection is expressed by the respective Cq value. No detection (nd).

Species	Sample location	Phylogenetic clade	Institute of Bee Health (Switzerland) Li et al. 2018 method			Istituto Zooprofilattico Sperimentale delle Venezie (Italy) Ward et al. 2007 method			Friedric Federal F Animal	h-Loeffler lesearch In Health (G	Institut, stitute for ermany)	WUR Biointeractions & Plant Health (The Netherlands)			
									Ward et al. 2007 method			Li et al. 2018 method (LNA modified)			
			Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	
A. tumida	Italy (Cosenza-Calabria)	A	22.99	22.21	22.74	35.32	35.38	35.25	29.29	28.34	28.21	27.63	27.44	27.54	
A. tumida	Burkina Faso (Bobo Dioulasso)	В	17.25	17.37	17.47	nd	nd	nd	nd	nd	nd	20.36	20.62	20.74	
A. tumida	Tanzania (Arusha)	В	20.55	20.31	20.18	21.76	22.06	22.41	22.23	21.59	22.63	24.33	24.23	24.44	
A. tumida	Philippines (Davao)	С	20.11	19.62	19.92	39.41	nd	nd	34.21	35.1	34.04	23.48	23.63	23.42	
A. flavicollis	South Korea	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
A. concolor	Australia	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
A. mellifera	Switzerland	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
-	Negative control (H,O)	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	

Statistical analyses

The SHB detection methods were pairwise compared using the Bland-Altman method comparison technique (Altman and Bland 1983) which tests the limits of agreement of two measurements of the same variable. The tests were performed using the NCSS 2022 Data statistical software. It provides the correlation coefficient and a diagnostic test to determine if the differences are normal (Test of normality of differences, Shapiro-Wilk, $\alpha = 0.05$).

Results

Comparison of detection methods

All methods were able to discriminate *A. concolor, A. inconspicua, A. flavicollis, A. major, A. mellifera* and *V. destructor* from *A. tumida*, implying that false positive results were not detected. In the case of hydrolysis probe methods (Ward et al. 2007; Li et al. 2018; Silacci et al. 2018), no amplification curves were observed above the auto-calculated threshold set by the qPCR software version (Bio-Rad CFX Maestro 1.0 Version 4.0.2325.0418). In the case of the SYBR Green qPCR detection method (Idrissou et al. 2018, modified), as the threshold was crossed by the *A. mellifera* samples (Cq values of 33.34 and 33.95), melting curve analyses were used to discriminate *A. tumida* samples from *A. mellifera*: *A. tumida* peak at Tm of 79.0– 79.5 °C whereas *A. mellifera* peak at Tm of 84.0–84.5 °C (Suppl. material 1: fig. S1).

However, the Ward et al. (2007) and the Silacci et al. (2018) methods produced some false negative results (Figs 1, 3, 4). Ward et al. (2007) did not detect beetles from Burkina Faso (Bobo Dioulasso) and Silacci et al. (2018) did not detect specimens collected from Burkina Faso (Bobo Dioulasso, Fada-Ngourma and Tenkodogo), Burundi (Rusiga) and Italy (Cosenza-Calabria). On the other hand, Li et al. (2018) and the modified Idrissou et al. (2018) effectively detected all specimens with no false negative results.

For the pairwise comparison between methods (Fig. 2), the Li et al. (2018) method was chosen as the reference because it showed lower variability of its Cq detection values (Fig. 1). The outliers under the inferior limit of agreement in all three comparisons (Fig. 2a, b, c) show that the Li et al. (2018) method detects the SHB samples at lower Cq values, significantly (Test of normality of differences, Shapiro-Wilk, p < 0.001 for all three comparisons).

Ring test using each participant's routine detection method

SHB DNA from single specimen collected in Italy (Clade A), Burkina Faso (Clade B), Tanzania (Clade B) and Philippines (Clade C) were tested blindly by each participant laboratory. The laboratories that used Ward et al. (2007) method failed to detect the SHB specimen from Burkina Faso, which accumulated 16 mismatches between primers and probe together (Suppl. material 1: fig. S2). In contrast, the laboratories using Li et al. (2018) method and its modified version (probe designed with Locked Nucleic Acid bases) were able to detect all of the tested specimens (Table 1). No interspecific cross detection was shown, neither for any of the used routine methods nor for any of the participant laboratories, on the DNA samples from specimens of *A. concolor, A. flavicollis* and *A. mellifera*.



qPCR detection method

Figure 1. Cq value (N = 83) distribution for the different small hive beetle qPCR detection method expressed in box, density and dot plots. A Cq value of 41 was assigned in case of no small hive beetle detection.



Figure 2. Bland-Altman pairwise method comparison. The vertical axis plots the Cq value differences between Li et al. (2018) and **a** Ward et al. (2007) **b** Silacci et al. (2018) and **c** Idrissou et al. (2018). The average Cq of the compared methods is plotted along the horizontal axis. The horizontal red line represents the mean of the differences. The blue horizontal lines define the limits of agreement using the z-value = 1.96 (95% CI).



Figure 3. Cq value distribution for each different small hive beetle qPCR detection method for specimen from the endemic African range. Red dash line represents Cq value 40, the limit of detection. Green dash line represents Cq value 30. A Cq value of 41 was assigned in case of no SHB detection. B: Benin, BF: Burkina Faso, Bu: Burundi, DRC: Democratic Republic of Congo, E: Ethiopia, Ke: Kenya, L: Liberia, Md: Madagascar, MW: Malawi, N: Nigeria, CAR: Central African Republic, SA: South Africa, SS: South Sudan, S: Sudan, Ta: Tanzania, U: Uganda. Initials of the site of collection in parentheses (i.e., Abo = Abomey).



Figure 4. Cq value distribution for each small hive beetle qPCR detection method for small hive beetle specimen collected from countries in the invasive range. Red dash line represents Cq value 40, the limit of detection. Green dash line represents Cq value 30. A Cq value of 41 was assigned in case of no SHB detection. A: Australia, BR: Brazil, CR: Costa Rica; Ca: Canada, Cu: Cuba, Ja: Jamaica, Me: Mexico, US: USA, Phi: Philippines, It: Italy; Po: Portugal. Initials of the site of collection in parentheses (i.e., Cai = Cairns).

Inter-laboratory comparison using the selected SHB detection method

From the results of the "Comparison of detection methods" section, the Li et al. (2018) method was chosen for the inter-laboratory SHB detection method comparison test. The proficiency of this method for the qPCR detection of SHB proved to be robust and reliable under each participating laboratory conditions. After performing the blind test, all of the SHB DNA samples, from different phylogenetic clades, were correctly confirmed by each participating laboratory (Table 2). Similarly to the previous assays, no interspecific cross detection (false positive results) was detected, for any of the DNA samples from specimens of *A. concolor, A. flavicollis* and *A. mellifera*.

The proficiency of Silacci et al. (2018) method for the qPCR detection of insect DNA (18S rRNA gene) was also tested to serve as an internal control for DNA quality and/or the presence of PCR inhibitors (e.g., ethanol). Insect DNA was detected in all specimens but not in some replications of the *A. concolor* specimen (Table 3).

Regarding the sensitivity of the Li et al. (2018) method, the highest sensitivity (positive detection of the three replicates with the lower DNA dilution) was detected at $5*10^{-6}$ ng/µl SHB DNA at the time the samples were freshly prepared. After the samples were delivered and tested under each participant laboratory conditions, detection of 100% of the samples for all laboratories was reached at $5*10^{-3}$ ng/µl SHB DNA (Table 4). This include samples that were unplanned and exposed to room temperature storage for 5 days due to delayed custom clearance during sample delivery.

Discussion

This study compared the effectiveness and specificity of the four DNA-based detection methods for SHB, *A. tumida*, published over the last 15 years. Our data clearly show that Li et al. (2018) and Idrissou et al. (2018) were the only methods that accurately detected all tested samples (N = 83) including 44 regions from Africa. This is the largest diversity of SHB specimens ever tested with those methods. The Idrissou et al. (2018) method was originally designed as an end point PCR method. However, this study proves that this method can be adapted for SYBR qPCR and

Table 2. Inter-laboratory comparison test of the Li et al. (2018) method for the PCR detection of SHB. Specimens of *Aethina tumida*,

 Aethina flavicollis, *Aethina concolor* and *Apis mellifera* were screened (blind test) by each participating laboratory. Positive detection is expressed by the respective Cq value. No detection (nd).

Species	Sample location	Phylogenetic clade	Institute of Bee Health (Switzerland)			Istituto Zooprofilattico Sperimentale delle Venezie (Italy)			Friedrich-Loeffler- Institut, Federal Research Institute for Animal Health (Germany)			WUR Biointeractions & Plant Health (The Netherlands)			
			Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	
A. tumida	Italy (Cosenza-Calabria)	A	22.99	22.21	22.74	27.93	28.27	28	32.99	32.6	29.51	27.63	27.75	27.53	
A. tumida	Burkina Faso (Bobo Dioulasso)	В	17.25	17.37	17.47	20.94	20.91	20	26.3	25.5	23.11	19.49	20.05	19.92	
A. tumida	Tanzania (Arusha)	В	20.55	20.31	20.18	25.55	25.19	25.52	26.21	27.56	29.09	24.36	24.59	24.74	
A. tumida	Philippines (Davao)	С	20.11	19.62	19.92	22.56	22.05	22.23	23.86	24.47	24.02	21.45	21.37	21.31	
A. flavicollis	South Korea	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
A. concolor	Australia	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
A. mellifera	Switzerland	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
-	Negative control (H ₂ O)	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	

Table 3. Inter-laboratory comparison test of the Silacci et al. (2018) method for the PCR detection of the insect's 18S rRNA gene. This method controls for amplifiable insect DNA. Specimens of *Aethina tumida, Aethina flavicollis, Aethina concolor* and *Apis mellifera* were screened (blind test) in each laboratory. Positive detection is expressed by the respective Cq value. No detection (nd).

Species	Sample location	Phylogenetic clade	Institute of Bee Health (Switzerland)			Istituto Zooprofilattico Sperimentale delle Venezie (Italy)			Friedrich-Loeffler- Institut, Federal Research Institute for Animal Health (Germany)			WUR Biointeractions & Plant Health (The Netherlands)		
			Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
A. tumida	Italy (Cosenza-Calabria)	A	19.23	18.69	18.50	27.31	27.19	27.17	27.63	28.26	26.23	21.91	21.97	21.91
A. tumida	Burkina Faso (Bobo Dioulasso)	В	15.43	16.27	16.63	25.11	25	24.22	24.36	26.65	24.67	18.45	18.71	18.66
A. tumida	Tanzania (Arusha)	В	24.63	24.25	24.23	31.24	31.25	31.11	30.51	30.32	31.25	24.11	24.38	24.35
A. tumida	Philippines (Davao)	С	27.31	26.63	26.74	32.56	32.03	32.13	33.48	33.39	33.23	26.27	26.40	26.15
A. flavicollis	South Korea	-	25.26	25.59	26.15	34.82	35.06	34.83	37.6	37.6	37.08	29.19	29.20	28.99
A. concolor	Australia	-	31.31	30.10	27.26	nd	nd	nd	nd	nd	43.99	36.62	35.09	36.33
A. mellifera	Switzerland	-	18.29	18.56	18.86	25.72	27.03	25.92	27.42	27.09	27.75	22.11	22.25	22.33
-	Negative control (H_2O)	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 4. Inter-laboratory sensitivity comparison test of the Li et al. (2018) method for the PCR detection of small hive beetles. Ten-fold dilutions of small hive beetle DNA were screened (blind test) in each laboratory. Positive detection is expressed by the respective Cq value (small hive beetle = SHB; No detection (nd)).

Sample name	DNA Concentration	Institute of Bee Health (Switzerland)			Istitu Speriment	to Zooprofil ale delle Ven	attico ezie (Italy)	Friedric Federal R Animal	h-Loeffler- lesearch In Health (G	Institut, stitute for ermany)	WUR Biointeractions & Plant Health (The Netherlands)		
	(IIg/µI)	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
SHB DNA dilution 1	5.00E-03	26.32	25.29	26.12	33.13	32.39	32.44	37.27	41.99	37.67	30.98	30.76	31.06
SHB DNA dilution 2	5.00E-04	29.52	29.62	29.79	36.38	36.29	35.58	nd	43.55	41.11	34.64	35.08	34.47
SHB DNA dilution 3	5.00E-05	33.82	32.35	33.17	nd	39.25	nd	nd	nd	nd	nd	39.21	39.04
SHB DNA dilution 4	5.00E-06	35.01	36.36	36.08	nd	nd	nd	nd	nd	nd	nd	nd	nd
SHB DNA dilution 5	5.00E-07	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
SHB DNA dilution 6	5.00E-08	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
SHB DNA dilution 7	5.00E-09	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Non-template control	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

can be used as an alternative more economic method because it does not require labelled probes compared to a qPCR hydrolysis method. From those two methods, the one published by Li et al. (2018) was slightly less variable regarding the detection thresholds (Cq values) and more importantly, having all specimens detected lower than Cq of 25, which is a clear indicator of the robustness of the method (Figs 1, 2).

No false negative results were observed with the Li et al. (2018) and the modified Idrissou et al. (2018) methods. The Li et al. (2018) method was able to confirm the identity of all SHB specimens even on individuals with 2 (Italy: Calabria; Ethiopia: Sidama; Congo: Lume) and 3 (Burkina Faso: Bobo Dioulasso and Tenkodoge) accumulated mismatches. Similarly, the Idrissou et al. (2018) method confirmed the identity of SHB specimens even on individuals with 3 (Uganda: Busiwu), 4 (Burkina Faso: Fada N'gourma) and 5 (Burkina Faso: Bobo Dioulasso) accumulated mismatches.

Regarding the sensitivity for each method, the comparison of the Cq values from the same samples across the methods provides hints for the robustness. A method can be considered more sensitive when the fluorescent amplification curve crosses the threshold at lower Cq values. Li et al. (2018) detection Cq values range from 13.26 to 24.07, while the modified Idrissou et al. (2018) Cq values range from 10.58 to 26.32 (Figs 1, 3, 4). However, for the Ward et al. (2007) and Silacci et al. (2018) methods, the sensitivity is much lower (Cq values above 30) compared to both Li et al. (2018) and modified Idrissou et al. (2018) methods (Figs 1, 3, 4). Moreover, for the samples with higher variability, which exceed the limits of agreement across the methods (Fig. 2), the Li et al. (2018) method showed to be the most reliable as it detects the SHB samples at lower Cq values.

Regarding the false negative results, they are intrinsically linked to the nucleotide mismatches between the sequences of the primers and probes against the target genome. The Ward et al. (2007) method was unable to detect a specimen from Burkina Faso (Bobo Dioulasso). This specimen accumulated 16 nucleotide mismatches in forward, reverse primers and probe all together (Suppl. material 1: fig. S2). Additionally, several specimens that were at the limit of detection showing high Cq values (Cq > 35) also accumulated several nucleotide mismatches (*i.e.* Burkina Faso: Tenkodoge (15 mutations), Burundi: Rusiga (8 mutations), Burkina Faso: Fada N'gourma and Congo: Lume (7 mutations); Fig. 3; Suppl. material 1: fig. S2). The Silacci et al. (2018) method seems to be more sensitive to mismatches. Depending of the mismatch nucleotide site, a single mutation in the probe is apparently able to produce a false negative *i.e.* Burundi (Rusiga). However, with mismatches at different nucleotide sites, the method was able to detect samples with 2 accumulated mismatches (*i.e.* Portugal and Philippines).

To validate those results, a blind ring test was conducted. Overall, the results matched what was previously observed when all methods were compared ("Comparison of detection methods" section). For example, in the blind ring test, the Wageningen University & Research (WUR) Biointeractions & Plant Health laboratory used the Li et al. (2018) method, with their own modifications in the probe (van Gent-Pelzer and Cornelissen 2021; Suppl. material 1: table S2), and did not produce any false positive or false negative results. In contrast, the results of the laboratories using the Ward et al. (2007) method were consistent with the reporting of the false negatives, which were observed for this particular method. The inter-laboratory comparison using the single chosen method (Li et al. 2018) showed a uniform consistence between all laboratories again without any false positive or false negative results. This result also shows that the method can be adapted to the operating differences in each laboratory (e.g., qPCR reagents, thermocycler types, operators).

The comparison of the various genetic detection methods allowed the evaluation of their strengths and limitations. The error types I and II are of particular importance. The evaluation showed that all methods performed with ideal accuracy regarding error type I, as no false positive was detected even when including several specimens from the genus *Aethina*. In contrast, there were differences in their performances regarding error type II as some SHB specimens were not detected by some methods. The Idrissou et al. (2018) and Li et al. (2018) methods have less sensitivity to nucleotide mismatches on the primer and probe's target sequences. The methods designed more recently performed better as more genetic information, in terms of more SHB specimen sequences, was available for the COI gene. This allowed for the design of primers and probes in regions with lower polymorphism or when certain polymorphic sites could not be avoided, degenerate nucleotides (W:A/T; Y:C/T) were also used (Li et al. 2018).
Conclusions

The evaluation of the molecular detection methods for SHB, clearly showed that both the Idrissou et al. (2018) and Li et al. (2018) methods avoid both false positives and negatives even when testing across the endemic and introduced regions. However, in view of its higher sensitivity among the tested methods, we propose to recommend the Li et al. (2018) method for the identification of SHB. Global application of such reliable molecular diagnostic tools will contribute to management and control efforts of this mandatory disease and invasive species.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization: MGP, OY, PN. Data curation: OY. Formal analysis: OY. Funding acquisition: PN. Investigation: OY, AG, MOS, MGP. Methodology: MOS, AG, OY, MGP. Resources: PN. Writing - original draft: PN, OY. Writing - review and editing: PN, AG, MOS, OY, MGP.

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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

Reliable molecular detection of small hive beetles

Authors: Orlando Yañez, Marga van Gent-Pelzer, Anna Granato, Marc Oliver Schäfer, Peter Neumann Data type: docx

- Explanation note: Country of origen of specimens, primers and probes, PCR protocols, melting curve analysis, nucleotide mismatches
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Research Article

Rising temperatures may increase fungal epizootics in northern populations of the invasive spongy moth in North America

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Abstract

Insect pest species are generally expected to become more destructive with climate change because of factors such as weakened host tree defences during droughts and increased voltinism under rising temperatures; however, responses will vary by species due to a variety of factors, including altered interactions with their natural enemies. Entomopathogens are a substantial source of mortality in insects, but the likelihood of epizootics can depend strongly on climatic conditions. Previous research indicates that rates of infection of the spongy moth (Lymantria dispar) by its host-specific fungal pathogen, Entomophaga maimaiga, increase with environmental moisture and decrease as temperatures rise. High temperatures may have direct and indirect (due to the associated drying) effects on the fungus, but the interactive effects between temperature and moisture level on larval infection are unclear. Here, we test the hypothesis that warmer, drier conditions will decrease rates of infection of spongy moth larvae by E. maimaiga. We evaluated the effects of precipitation and temperature on larval mortality caused by E. maimaiga with a manipulative field experiment, conducted in one of the northernmost and coldest parts of the spongy moth's non-native range in North America. We caged laboratory-reared spongy moth larvae in experimentally warmed open-air forest plots, exposing the larvae to soil inoculated with E. maimaiga resting spores during two consecutive trials. Caged larvae were exposed to three temperature treatments — ambient, 1.7 °C above ambient and 3.4 °C above ambient — and either supplemental precipitation (+173 mm per trial) or ambient precipitation. Opposite to our hypothesis, there was no significant effect of supplemental precipitation, nor an interaction between precipitation and temperature. There was, however, a significant positive effect of increasing temperature on the number of larvae infected. On average, in each respective trial, larval infection increased by 44% and 50% under the elevated temperature treatments compared to ambient temperature. Experimental warming may have increased infections because ambient temperatures at the field site were suboptimal for fungal germination. The results from this experiment suggest that, in colder portions of the spongy moth's invasive range, increasing temperatures due to climate change may enhance the ability of *E. maimaiga* to help control populations of the spongy moth.

Key words: Biological control, climate change, epizootiology, invasive species, spongy moth



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Introduction

Climate change is generally expected to amplify the overall impacts of forest insect pest species worldwide due to factors including increased voltinism and survival under higher temperatures, drought-induced weakening of tree defences and changes in foliage quality for defoliators (Jactel et al. 2019), although responses will vary amongst species (Pureswaran et al. 2018; Lehmann et al. 2020; Halsch et al. 2021) in part due to interactions with natural enemies (Frank 2021). Entomopathogens (i.e. bacteria-, fungi-, viruses- and nematodes-attacking insects) are extremely susceptible to changes in the environment, particularly temperature and moisture (e.g. precipitation, soil moisture, relative humidity) (Dara et al. 2019). These pathogens are major mortality agents of insects (Roy et al. 2009) and climate conditions that inhibit the transmission of pathogens may increase the probability that insect pest populations grow to outbreak levels (Skendžić et al. 2021). Most studies on the potential effects of climate change on host-pathogen systems have focused solely on the impacts of rising temperatures (Altizer et al. 2013; MacDonald et al. 2023). There are few manipulative field studies that test the independent and interactive effects of multiple abiotic variables on host-pathogen relationships. An exception is a manipulative microcosm study by van Doan et al. (2021) who found that elevated CO₂, increased temperature and decreased precipitation predicted by the extreme climate change scenario Representative Concentration Pathway 8.5 (RCP 8.5) did not directly compromise different natural enemies of herbivores. Their results suggest the potential for short term adaptations of natural enemies of herbivores to climatic change conditions (van Doan et al. 2021). As the effects of climate change are not limited to increasing air temperatures, we need to understand effects of other aspects of climate change, such as predicted increases in the frequency and severity of drought events (IPCC 2022), on insect pests and their pathogens (St. Leger 2021).

Increasing drought could have important implications for a host-pathogen interaction involving one of the most damaging forest pests in North America, the spongy moth (Lymantria dispar). Springtime drought may create favourable conditions for outbreaks of the spongy moth by inhibiting infections by the host-specific fungal pathogen Entomophaga maimaiga (Pasquarella et al. 2018). Results from laboratory and field observational studies have consistently demonstrated a positive effect of environmental moisture on germination of E. maimaiga resting spores, sporulation and infection of spongy moth larvae by both spore types-azygospores (resting spores) and conidia (Hajek et al. 1990b; Hajek and Soper 1992; Weseloh and Andreadis 1992b; Hajek and Humber 1997; Reilly et al. 2014). In addition, manipulative field experiments have shown that the addition of artificial rain increases rates of infection by both conidia and resting spores (Hajek and Roberts 1991; Weseloh and Andreadis 1992a; Smitley et al. 1995; Hajek et al. 1996), the latter needing water to germinate (Hajek and Humber 1997). High temperatures, especially in lab studies with constant exposure to \geq 30 °C, generally have a negative effect on E. maimaiga germination, sporulation and infection (Hajek 1999). However, results from field studies with exposure to variable temperatures are not consistent. For example, results from two-year observational field studies showed that increasing soil temperature can have opposite effects on E. maimaiga infection levels (Hajek and Humber 1997) or may have a positive effect in the first year and no effect in the following year (Siegert et al. 2008). In another observational field study, Reilly et al. (2014) found that mortality of field-collected larvae

due to *E. maimaiga* infections decreased with increasing temperature, but no clear relationship for laboratory-reared larvae exposed to field conditions. These past studies highlight the important role of weather for this host-pathogen relationship, but the effects of climate change remain unclear as no studies have investigated the independent and interactive effects of temperature and precipitation under field conditions. Additionally, most past field studies occurred in regions with more moderate climates compared to the coldest portions of the spongy moth's range (e.g. upper mid-western States, eastern Canada, Maine), making it difficult to extrapolate effects of weather across the *L. dispar* range.

Here, we experimentally tested the effects of temperature and precipitation on the relationship between the spongy moth and *E. maimaiga*. The spongy moth is a non-native defoliator of hardwood forests in north-eastern North America and causes approximately \$3.2 billion of damage per year in the United States and Canada (Bradshaw et al. 2016). *Entomophaga maimaiga* is the dominant source of spongy moth larval mortality (Liebhold et al. 2013; Hajek et al. 2015) and can cause high rates of mortality in both high and low-density spongy moth populations (Hajek et al. 1990a; Elkinton et al. 1991). As epizootics can occur in low-density host populations, it is possible that the fungus can slow or stop the invasive spread of spongy moth in North America by causing mortality along the insect pest's expanding range front.

The goals of this study were to understand whether and how predicted climate changes, specifically rising temperatures and increasing summertime drought (IPCC 2022), may affect spongy moth mortality caused by E. maimaiga infection. The effects of soil and plant canopy temperature and precipitation on larval infection initiated from resting spores were evaluated in a manipulative field experiment. Warmer and drier conditions are generally associated with lower infection levels because E. maimaiga resting spores require high soil moisture to germinate and infect larvae (Hajek 1999). Temperatures of 15-25 °C are optimal for resting spore germination and high temperatures, especially those ≥ 30 °C, can directly reduce infection levels (Hajek and Humber 1997). However, our experiment was conducted in one of the northernmost parts of the spongy moth's range in North America (Fig. 1), where typical springtime and early summer conditions may be too cold to support germination (Siegert et al. 2009). In cold ecoregions of the spongy moth's range (e.g. Mixed Wood Shield (MWS), Fig. 2), above-average temperatures would likely increase germination, whereas above-average temperatures may have the opposite effect in the warmest ecoregions (e.g. Southern United States Plains (SUP), Fig. 2, H₁). Therefore, we predicted that infection levels would increase under experimentally elevated temperatures; in other words, alleviation of low temperature metabolic limitation would overwhelm any effects of warmer temperatures on enhanced soil water deficits, especially under the cold temperature range being tested. To better test the sensitivity of fungal infection to moisture levels, we added supplemental precipitation and predicted that adding supplemental precipitation would increase infection rates (Fig. 2, H₂). Understanding the effects of these abiotic conditions in the north-westernmost portion of its U.S. range is important because spongy moth spread is occurring more rapidly in this region than anywhere else (National Slow the Spread (STS) Program 2022). Furthermore, a climatic suitability study predicted that this region would become more suitable for the spongy moth under a 1.5 °C increase in mean daily temperature compared to historical averages (Gray 2004).



Figure 1. Location of the experimental site. The star represents the Hubachek Wilderness Research Center near Ely, Minnesota. Hatched area represents the non-native, established range of the spongy moth in the United States. Solid beige areas represent the spongy moth regulated areas in provinces and territories in Canada. In Canada, the entire Province of Quebec is regulated for the spongy moth, but the pest is only found in southern Quebec. Data for the spongy moth range in the United States were sourced from the USDA APHIS PPQ spongy moth quarantine records (National Slow the Spread (STS) Program 2022). Data for the regulated areas of Canada were sourced from the Canadian Food Inspection Agency (CFIA 2020).

Materials and methods

Study system

The infection cycle of *E. maimaiga* begins in the spring, when overwintering resting spores in the forest soil begin to germinate approximately 1-2 weeks before spongy moth larvae emerge (Hajek and Humber 1997). Resting spores are always responsible for the primary infection cycle of the season, which occurs when infectious germ conidia are released from germinating resting spores. These germ conidia can become airborne and, when they land on spongy moth larvae, they germinate, infect and then kill a larva in approximately five days (Hajek et al. 1995). Prior to larval death, E. maimaiga grows inside of the insect and, after host death, it grows outwards through the cuticle and produces and ejects thousands to millions of new conidia (Shimazu and Soper 1986; Hajek et al. 1993). Infections initiated by germ conidia released from resting spores (e.g. the primary infection cycle) only produce conidia, never resting spores (Hajek 1997). Conidia released from dead spongy moth larvae (cadavers) are responsible for all subsequent infection cycles during that season. When spongy moth larvae reach later instars (5th-6th), infected larvae begin to produce resting spores as opposed to conidia (Hajek and Shimazu 1996). The exact environmental cues



Figure 2. Hypothetical response curves of *Entomophaga maimaiga* resting spore germination. Each curve represents how resting spore germination would likely respond to different temperature and precipitation conditions during the spongy moth larval period (spring-time). The points on the average precipitation curve represent five ecoregions that are within the spongy moth's range in the North America— Mixed Wood Shield (MWS), Mixed Wood Plains (MWP), Central United States Plains (CUP), Appalachian Forest (AF) and Southern United States Plains (SUP) — based on the Environmental Protection Agency's Level II Ecoregions (Omernik 1987; Omernik and Griffith 2014). The Hubachek Wilderness Research Center is located in the MWS ecoregion.

that cause spore production to switch from conidia to resting spores are unknown, but temperature and larval instar are both contributing factors (Hajek and Humber 1997; Hajek 1999). Resting spores produced in later instar spongy moth cadavers go dormant and overwinter, adding to the reservoir of resting spores in the soil. Resting spores are typically dormant for 1–2 years prior to germination and can survive for at least 6 years in the field (Weseloh and Andreadis 1997; Hajek et al. 2004; Hajek et al. 2018).

Manipulative field experiment

The experiment was conducted in 2022 at the University of Minnesota's Hubachek Wilderness Research Center (HWRC; (47.9481, -91.7583) near Ely, Minnesota. The HWRC is in the north-westernmost portion of the spongy moth's expanding range front (Fig. 1), at an elevation of 415 m a.s.l. We used experimentally warmed forest (closed canopy) plots that were part of a long-term climate change experiment, the Boreal Forest Warming at an Ecotone in Danger (B4WarmED) project (Rich et al. 2015). The plots were within 40- to 60-year-old stands of mixed aspen — birch — fir on coarse-textured upland soils. Tree saplings of various species that were planted for the B4WarmED project, natural understorey herbs and shrubs, as well as fallen leaves and moss, were generally found within each plot (Reich et. al 2022). The open-air plots were simultaneously warmed aboveground and belowground via ceramic heating lamps and buried resistance-type heating cables, respectively (Rich et al. 2015).

Larvae for the field experiment were reared from eggs obtained from a disease-free spongy moth colony from the United States Department of Agriculture's Forest Pest Methods Laboratory (Buzzards Bay, MA). Larvae were reared on an artificial diet (Bell et al. 1981) to the 4th instar in an environmental chamber at 25 °C, 70% relative humidity and 15 hours of light/d. We used 4th instar larvae for the experiment in alignment with established procedures (Reilly et al. 2014). To align the timing of the experiment with when wild spongy moths at the field site would be in the 4th instar, we estimated when wild larvae would reach this stage in 2022 using the spongy moth phenology model, which consists of a suite of temperature-dependent sub-models (Régnière and Sharov 1997, 1998, 1999; Gray et al. 2001). We conducted replicate trials when 5%, 50% and 95% of larvae at HWRC were predicted to have reached the 4th instar. Matching the trials with larval phenology also helped ensure the experiment aligned with E. maimaiga's resting-spore germination period, which begins approximately 2 weeks before larval emergence and ends when spongy moth larvae reach later instars (e.g. 5th-6th instars) (Hajek and Humber 1997).

To ensure the presence of *E. maimaiga* in the study plots, we inoculated the plots in the summers of 2019 and 2021 with cadavers of spongy moth larvae that contained resting spores. The 2019 inoculation event was necessary because spongy moth densities were likely very low at the field station, as determined from data from the Slow the Spread (STS) Foundation (National Slow the Spread (STS) Program 2022). Due to the low spongy moth densities at HWRC, it was uncertain whether E. maimaiga was already present at HWRC. Originally, the experiment was planned for summer 2020, but the COVID-19 pandemic prevented the experiment from occurring 2020-2021. As some spores released in the 2019 inoculation event likely germinated (without reproducing) in the subsequent seasons, an augmentative 2021 inoculation event was necessary to ensure enough resting spores were present in 2022 when the experiment occurred. Based on our resting spore release estimates of 3.6×10^6 and 4.0×10^6 resting spores per cage site in 2019 and 2021, respectively (Hajek and Humber 1997; see Supplementary file 1: Details on soil inoculation procedure with *Entomophaga maimaiga*), the number of resting spores added to the soil at each cage site by the year 2022 was estimated to be 7.6×10^6 . This estimate considers that all of the resting spores from the 2021 inoculation were present and viable in 2022.

For the inoculation events, we obtained resting spore-filled cadavers from Massachusetts and Virginia, U.S. when *E. maimaiga* epizootics were ongoing. The cadavers were found hanging upside down on the trunks of trees with prolegs extended at 90°, a visual cue that demonstrates death from infection by the fungus (Blackburn and Hajek 2018). The inoculations were carried out using procedures previously shown to be effective (A. Diss-Torrance personal correspondence; Hajek et al. 2021). In each inoculation year, first, we ground the field-collected spongy moth cadavers in a food processor and divided the ground material into 36 equal portions (0.54 grams each), one for each experimental plot. We then mixed each portion with 30 g of sterile potting soil. For each inoculation, we spread the mixture on top of the forest soil in the same two randomly selected locations in each plot. Each inoculation site was roughly 31×23 cm²; the sites were where we would place caged spongy moth larvae during the experiment. Additional details about the inoculation procedures are provided in Supplementary file 1: Details on soil inoculation procedure with *Entomophaga maimaiga*. The effects of temperature on the infection of spongy moth larvae by *E. maimaiga* were examined by caging larvae within 7.1 m² circular plots that were assigned to three different temperature treatments (ambient temperature, 1.7 °C above ambient and 3.4 °C above ambient). However, we note that the cages and the larvae experienced approximately 5–10% lower temperature increases than the overall temperature increases of 1.7 °C and 3.4 °C above ambient achieved by the system (for more details on the system performance and achieved temperatures see Rich et al. (2015)). The plots were arranged in a block design with two replicate plots of each temperature treatment in each of the three blocks (Fig. 3). Plots within a block were separated by 4–13 m and were 8 m apart on average. The blocks were spaced approximately 15–23 m from each other, with an average separation of 19 m, within the same closed canopy habitat. Temperature was manipulated in the plots annually from approximately the beginning of April until the end of November every year since 2009.

Caged larvae were exposed to temperature and precipitation treatments in three separate four-day Trials; 30 June to 04 July (trial one); 06 July to 10 July (trial two); 12 July to 16 July (trial 3). We introduced simulated rainfall to one of two cages per plot (randomly selected), creating two precipitation treatments: ambient and supplemental (Fig. 3). To determine an appropriate amount of additional, simulated precipitation, we obtained long-term daily precipitation records from two nearby NOAA weather stations (https://www.ncdc.noaa.gov/) in Ely, Minnesota, which, together, provided coverage from 2000 to 2021 (USC00212561 47.9056, -91.8283; USC00212543 47.9239, -91.8586). From these data, we calculated the long-term (2000–2021) mean and standard deviation (SD) of precipitation for the date range of the experiment, 30 June to 17 July. The total volume of water added was equal to 1 SD of the long-term mean, with this volume calculated as:

water volume $(ml) = catchment area (cm²) \times rainfall depth (mm)$ (1)

where catchment area was the area of a single cage $(31 \times 23 \text{ cm}^2)$ and rainfall depth was 1 SD of the long-term mean of 7 mm. The total volume of water added to a cage receiving supplemental precipitation was 518 ml. The rationale for choosing to supplement precipitation by this amount (1 SD of the long-term mean for the duration of the experiment) was that we wanted to substantially increase soil moisture without increasing the total amount of precipitation (ambient + supplemental) by an amount that was unusually high in recent history. We applied one-third of the total volume of water during each four-day trial, with half of the water added at dusk on the first and third days of each trial by sprinkling with a watering can. To reduce confounding effects from water contaminants (e.g. minerals, pollutant, etc.), we used deionised water.

We tested the effects of temperature and precipitation on fungal infection by deploying two cages, each containing 19 early 4th-instar larvae, in each plot; for a total of 684 larvae per trial (Figs 2, 3). The cages were made by folding aluminium window screening to a dimension of 31×23 cm², with a 2-cm high interior cavity, after which the seams were stapled and taped closed (Hajek and Humber 1997; Reilly et al. 2014; Fig. 4). To ensure contact with the soil, we brushed aside the leaf litter at each cage site and then installed the cages flush with the soil. Each cage was protected from vertebrate predators and weather-related hazards (e.g. fallen limbs) by a covering box of 12-mm² hardware mesh and both the cage and cover were anchored to the soil with landscape staples (Reilly et al. 2014; Fig. 4). Two 0.5 g cubes of artificial wheat germ diet (Bell et al. 1981) were provided in every cage.



Figure 3. Diagram of one experimental block. Within each of three experimental blocks, we installed two cages of spongy moth larvae per plot (figure adapted from Rich et al. (2015)).



Figure 4. The cage design used to expose larvae to *Entomophaga maimaiga* resting spores in the soil (Reilly et al. 2014).

Following each field trial, we transferred the larvae to the lab and secured them individually in lidded 30 ml plastic cups containing a 0.20 g piece of artificial wheat germ diet (Blackburn and Hajek 2018). We maintained larvae at 18–22 °C and monitored them for 10 days or until death, whichever occurred first (Blackburn and Hajek 2018). Larvae that died within the 10-day monitoring period were placed on 1.5% water agar plates and checked daily for 3 days for conidial production (Hajek et al. 1990b). Although conidia are often visible without magnification, we noticed during the first trial's observation period that conidia were sometimes only apparent under a dissecting microscope. We did not analyse trial one results because of the possibility that we missed conidia on cadavers that we inspected without a dissecting microscope, as conidia are very short lived. We examined cadavers from trials two and three under both a dissecting and a phase contrast microscope. For phase

contrast microscopy, we macerated and smeared a larva on a microscope slide and observed the specimen at 200–400× magnification (Blackburn and Hajek 2018). We counted a larva as infected by *E. maimaiga* if we saw conidia either externally or via phase contrast. It is also possible for larvae to have resting spores present instead of, or in addition to, conidia (Hajek 1999), but we did not expect this because previous research indicated that infections initiated by germ conidia from resting spores only produce conidia, not resting spores (Hajek 1997).

To understand how the weather conditions prior to and during the trials compared to average climatic conditions, we obtained long-term climate data for 2000 to 2022 from the same two NOAA weather stations used to calculate the amount of supplemental precipitation. Plot-level daily data on soil moisture for 20 June to 17 July was measured via automated, permanently installed water reflectometers (Model CS616 from Campbell Scientific), with one reflectometer per plot that collected measurements on an hourly interval for the top 30 cm soil profile.

Data analysis

We tested the hypotheses that temperature, precipitation and their interaction would affect the number of spongy moth larvae infected (and almost certainly killed) by E. maimaiga. We built a model with these variables and also included a fixed effect of block and random effect of plot. Including plot as a random effect allowed for the model to account for variance amongst plots, for example, potential non-independence of values within a given plot (Crawley 2013). Block was modelled as a fixed effect because there were only three levels in block and estimates of variance of random effects with so few levels are inaccurate (Arnqvist 2020). A linear mixed effects model with all of these variables was over-parameterised. Therefore, we assessed that block or plot could be dropped without adversely affecting model parsimony (fit balanced by a penalty for the number of parameters), with parsimony quantified using AIC values. Models with $\Delta AIC < 2$ were considered to have substantial support (Burnham and Anderson 2002), where Δ AIC of model *i* equal to AIC_{*i*} – min(AIC) and min(AIC) is the AIC of the highest-ranking model. For both trials, we selected the model that was not over-parameterised, had substantial support ($\Delta AIC < 2$) and the fewest number of variables dropped (See Suppl. material 2: Model selection using AIC scores). For both trials, a model that included block, but not plot was selected (Suppl. material 2). We assessed the effects of temperature, precipitation, the temperature × precipitation interaction and block on the number of infected larvae using generalised linear models (GLM). For each model, we specified a Poisson distribution and a log link. We performed all statistical analyses in R (R Core Team 2022). The models were fitted using the 'glmer' function of the 'lme4' package (Bates et al. 2015). To conduct pairwise comparisons, we performed Tukey's tests using the package 'emmeans' (Lenth 2022).

Results

Baseline climatic and abiotic conditions at the field site

Compared to the long-term mean from 2000 to 2021, NOAA weather station data (USC00212561 47.9056, -91.8283; USC00212543 47.9239, -91.8586) indicated that mean daily minimum and maximum temperatures during this experiment were

near-average and slightly below-average, respectively (Table 1). Air temperature gradually increased over the course of the experiment; the average minimum and maximum daily air temperatures recorded by the NOAA weather stations during the second trial were 11.4 °C and 23.5 °C; and 12.4 °C and 24.5 °C during the third trial.

Mean daily precipitation during the experiment was 72% lower than the longterm mean (Table 1). However, prior to the experiment, mean daily precipitation in May was 38% higher than the long-term average for this month (Table 1). Soil moisture decreased during the experiment and, on the last day, average soil moisture across all plots was 0.18 Θ (cm³H₂O cm⁻³soil), compared to 0.25 Θ at the start of the experiment. On average, plots under the high temperature treatment (3.4 °C above ambient) had the driest soil ($\Theta = 0.20 \pm 0.01$) and plots under the low (1.7 °C above ambient) and ambient temperature treatments had similar soil moisture, $\Theta = 0.24 \pm 0.02$ and $\Theta = 0.23 \pm 0.02$, respectively. Soil moisture in the region of the supplemental precipitation was not measured.

Effects of temperature and simulated precipitation on numbers of infected larvae

Increasing temperature had a significant, positive effect on the number of infected larvae in the second (P = 0.004) and third (P = 0.001) trials (Table 2; Fig. 5). In trial two, averaging across the precipitation treatments, the numbers of infected larvae were 24% and 65% higher in cages where temperature was elevated by 1.7 °C and 3.4 °C, respectively, compared to cages in plots at ambient temperature (Fig. 5). In trial three, the numbers of infected larvae were 56% and 44% higher under temperatures elevated by 1.7 °C and 3.4 °C, respectively, compared to ambient temperature. In both trials, very few infections occurred under ambient temperatures (Fig. 5). There were no significant effects of supplemental precipitation or interactive effects of temperature and precipitation or block on number of infected larvae in either trial (Table 2).

Table 1. Climate data for 2000 to 2022, sourced from NOAA stations USC00212561 and USC00212543 (Ely, Minnesota; https://www. ncdc.noaa.gov/). Values reported are mean daily averages ± standard deviation, during the dates of the experiment (30 June – 17 July) for the long-term (2000–2021) and in 2022. Precipitation is also reported during May because above-average rainfall in 2022 may have influenced *Entomophaga maimaiga* germination.

Year	May	30 June – 17 July			
	Precipitation (mm)	Minimum temperature (°C)	Maximum temperature (°C)	Precipitation (mm)	
Long-term mean (2000 to 2021)	2.5 ± 5.4	12.7 ± 3.1	26.7 ± 4.2	0.9 ± 1.5	
2022	4.0 ± 8.2	12.3 ± 3.1	24.3 ± 2.4	3.3 ± 7.1	

Table 2. Results from generalised linear models on effects of temperature and precipitation on infection of spongy moth larvae by *Ento-mophaga maimaiga* in trials two and three.

Trial	Explanatory Variable	Degrees of Freedom	Chi-Square	P value
2	Block	2	2.6	0.272
	Precipitation	1	0.22	0.637
	Temperature	2	11.23	0.004
	Temperature × Precipitation	2	4.45	0.108
3	Block	2	3.67	0.16
	Precipitation	1	0.25	0.617
	Temperature	2	13.23	0.001
	Temperature × Precipitation	2	0.91	0.634



Figure 5. Results of manipulative field experiment. Effect of temperature and precipitation treatments on number of spongy moth larvae infected by *Entomophaga maimaiga* (mean ± SE) in (a) trial two and (b) trial three. The precipitation treatment consisted of adding +173 mm water to each cage over the course of a trial.

Discussion

Multiple studies have reported positive effects of environmental moisture on spongy moth larval infections by *E. maimaiga* (Hajek 1999; Reilly et al. 2014), but using a manipulative field experiment, we found that only the temperature treatments, not supplemental precipitation, affected larval infections by this pathogen. We found that larval infections increased where temperatures were elevated above ambient. This finding aligns with our prediction, based on a climate suitability study (Siegert et al. 2009), that cold temperatures may inhibit *E. maimaiga* in the north-westernmost portions of the spongy moth's invasive range. These results suggest that rising temperatures associated with climate change may increase larval mortality by *E. maimaiga* in cold portions of the spongy moth's range (e.g. Minnesota, northern Wisconsin and eastern Canada).

Contrary to our predictions, supplemental precipitation and its interaction with temperature, did not affect the number of larvae that became infected by the fungus. This finding was surprising given that multiple past studies have found positive associations between rainfall and larval infections from resting spores (Weseloh and Andreadis 1992a; Weseloh and Andreadis 1992b; Hajek et al. 1996; Reilly et al. 2014). One potential explanation for the lack of a precipitation effect in this study is that moisture requirements for resting spore germination were fulfilled before the experiment began, given that precipitation was above average during the month of May prior to the start of the experiment (Table 1). Supporting this possibility, a past study modelled the relationships between rainfall, temperature and the phenology of infection and found that the timing of rainfall, not just amount of rainfall, is a critical factor influencing infection rates (Weseloh et al. 1993). Additionally, using empirical data on *E. maimaiga* epizootics, Hajek et al. (2015) found that infection increased significantly with May rainfall, whereas the relationships between infection and precipitation in June and April were negative and not significant, respectively. Future field experiments that incorporate a

reduced-moisture treatment along with an increased-moisture treatment could help resolve how infection levels will change with increasing drought severity.

Results from this study highlight the importance of considering geographic location when assessing the impacts of temperature and moisture conditions on larval infection. The general consensus of past research on the role of weather for this host-pathogen relationship is that warmer and drier conditions inhibit infections by *E. maimaiga* (Hajek 1999). Prior field studies on the effect of temperature on the spongy moth-*E. maimaiga* interaction were conducted in areas with warmer climates compared to the present study (Hajek et al. 1996; Hajek and Humber 1997; Reilly et al. 2014), which may explain why the positive effect of increasing temperature found in this experiment contrasts with that found in warmer regions. For example, mean daily air temperatures during the spongy moth's larval period are consistently lower in Ely, Minnesota (USC00212555 47.9746, -91.4495) compared to other portions of the spongy moth's non-native range for example, Ithaca, New York (USC00304174 42.4491, -76.4491); Oakland, Maryland (USC00186620 39.4131, -79.4003); and Gassaway, West Virginia (USC00463361 38.6649, -80.7672) (Fig. 6).

While epizootics have occurred during years that were warmer and drier than average (Hajek et al. 1996), host densities must be high enough to offset the negative effects of reduced fungal germination under these abiotic conditions. A past modelling study showed that the threshold host density at which epizootics occurred changed under different abiotic environmental conditions (Kyle et al. 2020). Fungal density is also an important determinant of epizootics and, together, the interaction between host and fungal densities with weather drive epizootics (Hajek and Shapiro-Ilan 2018). While often overlooked, fungal density may be critical to understanding why epizootics can occur even when host densities are low.

Certain caveats should be considered in extrapolating our findings to the effects of climate on the host-pathogen interaction between the spongy moth and *E. maimaiga*. First, like many experimental studies on the spongy moth (Williams et al.



Figure 6. Mean daily temperature at different locations within the spongy moth's non-native range. Locations include: Ithaca, New York (USC00304174 42.4491, -76.4491); Oakland, Maryland (USC00186620 39.4131, -79.4003); Gassaway, West Virginia (USC00463361 38.6649, -80.7672); and Ely, Minnesota (USC00212555 47.9746, -91.4495). Temperature data are from 2000–2022 (https://ncdc.noaa.gov/). Ely, Minnesota (MN) is the weather station nearest to the experimental site. The lines are dashed during the spongy moth's larval period at each location, based on phenology model predictions (Régnière and Sharov 1997, 1998, 1999; Gray et al. 2001).

2003; Mason et al. 2014), our study was conducted using spongy moths obtained from USDA's Forest Pest Methods Laboratory and reared using artificial diet. It is not known whether this lab strain and wild spongy moths differ in their susceptibility to infection by *E. maimaiga* or if larval diet influences susceptibility to this pathogen. Therefore, wild spongy moths under the same conditions may be infected at lower or higher rates. Second, because there is evidence that temperature can alter rates of larval infection (i.e. susceptibility; Shimazu and Soper (1986)) by the fungus, we cannot rule out the possibility that changes in larval susceptibility, rather than changes in fungal germination and sporulation, influenced the observed temperature-driven changes in rates of infection.

Conducting multi-year manipulative experiments that assess the effects of annual variability in weather on this host-pathogen relationship could help quantify the range of temperature and precipitation conditions under which larval infection rates may be high enough to spark epizootics. If rising temperatures lead to increased infections of *E. maimaiga* in the colder regions of the spongy moth's range in North America, as is suggested by the results of this experiment, it is possible that rates of spongy moth range expansion in these regions could decrease in the future. However, temperatures in the north-westernmost portion of the spongy moth's range are expected to become more suitable for the pest under a 1.5 °C warming scenario (Gray 2004). Therefore, the overall effects of climate warming on spongy moth populations in these colder ecoregions is unclear. To provide information for management decisions on slowing the spread of spongy moth, particularly in the north-westernmost region of this non-native range, where rates of range expansion are fastest, it is imperative that we untangle the independent and interactive effects of temperature and precipitation on the relationship between the spongy moth and E. maimaiga. Doing this will help managers identify when and where climatic conditions are expected to inhibit or enhance larval mortality due to E. maimaiga, which in turn, would provide information for decisions on employing methods to slow the spread of the spongy moth.

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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: KJH, PBBR, CAR. Data curation: AS, CAR. Formal analysis: CAR. Methodology: HN, KJH, AEH, PBBR, AS, CAR. Project administration: CAR, KJH, PBBR. Supervision: KJH, AEH. Visualization: CAR. Writing - original draft: CAR. Writing - review and editing: KJH, AEH, PBBR, CAR, HN, AS.

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Data availability

The data underpinning the analysis reported in this paper are deposited in the Dryad Data Repository at https://doi.org/10.5061/dryad.44j0zpcp4.

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Supplementary material 1

Details on soil inoculation procedure with Entomophaga maimaiga

Authors: Clare A. Rodenberg, Ann E. Hajek, Hannah Nadel, Artur Stefanski, Peter B. Reich, Kyle J. Haynes

Data type: pdf

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Supplementary material 2

Model selection using AIC scores

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Research Article

Performance in the recruitment life stage and its potential contribution to invasive success in the polyploid invader *Centaurea stoebe*

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Abstract

The recruitment life stage, including germination and early seedling establishment, is the most vulnerable life stage of plants and has cascading effects on plant performance at later life stages. However, surprisingly little is known on the eco-evolutionary processes that determine the success of biological invasions at this life stage.

We performed germination experiments with and without simulated drought stress and monitored early seedling growth in diploid and tetraploid *Centaurea stoebe*. While diploids are the major cytotype in the native European range, only tetraploids became invasive in North America. Thus, *C. stoebe* is an excellent model species to simultaneously study both, pre-adaptive differences in the native range (diploids vs. tetraploids) and post-introduction evolution in the non-native range (native tetraploids vs. non-native tetraploids). To account for broad spatial-environmental variation within cytotypes and ranges, we germinated 23,928 seeds from 208 widely distributed populations. Citation: Kožić K, Hartmann M, Callaway RM, Hensen I, Nagy DU, Mráz P, Al-Gharaibeh MM, Bancheva S, Diaconu A, Danihelka J, Ensing DJ, Filep R, Gudžinskas Z, Hajdari A, Nicoară R, Lachmuth S, Moffat CE, Novikov A, Purger D, Slate ML, Synowiec A, Vonica GD, Zuleger AM, Rosche C (2024) Performance in the recruitment life stage and its potential contribution to invasive success in the polyploid invader Centaurea stoebe. NeoBiota 95: 309-329. https://doi. org/10.3897/neobiota.95.127654 Tetraploids germinated better than diploids. Within tetraploids, invasive populations outperformed native populations in germination. However, these differences were not evident under simulated drought stress. Seedlings of invasive tetraploids had a higher biomass and developed the first true leaf earlier than those from the native range, while the native cytotypes did not differ in these early seedling traits.

Our results suggest that a combination of pre-adaptation related to superior performance of polyploids (greater and faster germination) and post-introduction evolution towards higher performance in the invasive range (greater and faster germination, greater and faster accumulation of seedling biomass) may have contributed to the invasion success of tetraploid *C. stoebe* in North America.

Key words: Biological invasions, Cox-regression, geo-cytotypes, germination, pre-adaptation, post-introduction evolution, seedling establishment traits, spotted knapweed

Introduction

Germination and early seedling establishment comprise the recruitment life stage, which is likely the most vulnerable life stage of plants (Donohue et al. 2010; James et al. 2011; Winkler et al. 2024). Moreover, performance in the recruitment life stage has cascading influence on fitness, survival and competitive ability at later life stages (Donohue et al. 2010; Gioria and Pyšek 2017; D'Aguillo and Donohue 2023). Germination and early seedling establishment are particularly important for non-native plants because they are mostly introduced as seeds and their spread is often associated with effective dispersal and fast colonization of empty niches in ruderal habitats (Colautti et al. 2006; Pyšek and Richardson 2007; Theoharides and Dukes 2007). However, surprisingly little is known about the eco-evolutionary processes determining invasive success in the recruitment life stage (Jiménez-Alfaro et al. 2016; Saatkamp et al. 2019; Larson et al. 2020). In particular, we need more information on whether germination and early seedling traits exhibit 1) pre-adaptive differences between successful vs. non successful invaders (van Kleunen et al. 2010), and 2) differences between native vs. non-native populations due to post-introduction evolution (Parker et al. 2013).

With regard to pre-adaptation, polyploidy has been increasingly recognised as an advantage for invasion success because polyploid plants are more likely to become invasive than closely related diploids (Pandit et al. 2011; te Beest et al. 2012). Polyploids have, on average, higher competitive ability and fecundity than diploids (reviewed in Rutland et al. 2021) and they are also assumed to exhibit greater colonization success in ruderal environments (van Drunen and Johnson 2022). However, it remains unclear whether polyploidy confers benefits during the recruitment life stage, and if so, whether this may contribute to explaining the apparent overrepresentation of polyploids among invasive plants. The best models to test these relationships are polyploid complexes where the polyploid cytotype is more successful in the non-native range than its diploid relative (reviewed by te Beest et al. 2012).

Polyploids are also assumed to be better pre-adapted to stressful environments and show higher phenotypic variation than diploids (Chen 2007; Rice et al. 2019). More specifically, polyploids often exhibit faster germination under ambient conditions. This is especially important during range expansions, because fast germination and seedling growth could be advantageous to promote spread in ruderal habitats (Harper et al. 1970). At the same time, polyploids may show a more conservative germination behaviour than diploids under unfavourable conditions (e.g., delaying germination while drought). Such conservative germination behaviour should increase their overall niche tolerance compared to diploids (Leitch and Leitch 2008; Stevens et al. 2020; Rutland et al. 2021) and ultimately increase survival probabilities of the emerging seedling (Saatkamp et al. 2019; Larson et al. 2020). However, studies on polyploid complexes in the context of biological invasions have not yet made this comparison. Therefore, we need to understand how germination differs between native and non-native populations under optimal and suboptimal conditions. With respect to post-introduction evolution, polyploidy may also enhance the potential for rapid evolutionary response to changing environmental conditions (Nagy et al. 2018; van Drunen and Johnson 2022). This is important for non-native species as they often experience climatic niche shifts (e.g., Bates and Bertelsmeier 2021) and encounter novel biotic interaction partners (e.g., Shah et al. 2014; Sheng et al. 2022) in their non-native ranges. Such altered environmental conditions may cause rapid post-introduction evolution, e.g., towards high-performance genotypes in the non-native ranges (Matesanz and Sultan 2013; Villasor et al. 2024), but information is lacking on whether this is also the case in the recruitment life stage (Nagy et al. 2024).

Finally, it is important to consider that populations from native and non-native ranges can show a pronounced among-population variation within either range (Rosche et al. 2019; Lucas et al. 2024). This variation should be associated with the population histories of the study populations such as the habitat type of source populations (e.g., between ruderal vs. (semi-)natural habitats) or may differ along climatic gradients such as aridity regimes in the population history (Baskin and Baskin 1998; Donohue et al. 2010; Al-Gharaibeh et al. 2024; Nagy et al. 2024). For example, more pronounced fluctuations in water availability in ruderal than (semi-)natural habitats may select for fast germination and seedling growth with a high investment in root mass allocation to quickly occupy open germination niches (Chapin et al. 1993; Larson et al. 2020). Nevertheless, most studies that investigated post-introduction evolutionary changes considered relatively few populations and underestimated variation within native and non-native ranges (reviewed by Lucas et al. 2024). Many studies have also examined different environmental gradients in native vs. non-native ranges, which may lead to false-positive differences between the ranges in the traits under investigation (reviewed by Colautti and Lau 2015). Similar to the study of post-introduction, assessing pre-adaptive differences between cytotypes requires a broad and representative sampling of populations within each cytotype (Rosche et al. 2018).

An example of a polyploid plant invader that shows evidence for post-introduction evolution is *Centaurea stoebe* L. (spotted knapweed; Asteraceae), a polyploid complex consisting of a diploid and a tetraploid cytotype. Both cytotypes are native to Europe, whereas only tetraploids are invasive in North America (Mráz et al. 2011). This cytotype shift between the ranges results in three so-called geo-cytotypes (GCTs): native European diploids (EU2x), native European tetraploids (EU4x) and non-native North American tetraploids (NA4x). Thus, *C. stoebe* is a suitable model to simultaneously test both pre-adaptive differences within the native range (EU2x vs. EU4x) and post-introduction evolution in the non-native range (EU4x vs. NA4x). Previous research found similar germination rates between the cytotypes (Hahn et al. 2013), but greater seedling root growth rates in tetraploids than diploids (Collins et al. 2013). Although these two studies provided important insights into the recruitment ecology of this polyploid complex, they did not involve broad spatial-environmental gradients along the distribution ranges of the three GCTs, nor did they identify drivers of among-population variation within each GCT. There is also no information on whether the germination behaviour of GCTs differs across treatments, and there are no data on potential post-introduction evolution in early seedling traits between NA4x vs. EU4x.

Here, we studied germination and early seedling traits in 208 Centaurea stoebe populations that occupy a wide environmental variation in local aridity regimes across the three GCTs. We also differentiated between populations from ruderal vs. (semi-)natural habitats. We performed germination experiments with and without simulated drought stress and monitored early seedling development. Our study was directed by the following hypotheses: EU4x shows a greater performance in recruitment traits than EU2x which would indicate pre-adaptive differences to successfully invade North America; 2) NA4x outperforms EU4x in recruitment traits which would suggest evidence of post-introduction evolution in the non-native range; and 3) differences in recruitment traits can be explained by the population history (e.g., ruderal populations germinate faster than (semi-) naturals; mesic populations show higher germination than arid populations) which would identify drivers of among-population variation within the GCTs. By understanding these mechanisms, we expect to reveal factors that contribute to the successful invasion of tetraploid C. stoebe which could have broader implications for predicting future invasion dynamics. More generally, we anticipate to gain insights into the significance of the recruitment life stage in the context of biological invasions and how polyploidy can confer benefits during this life stage.

Materials and methods

Study species

Taxonomically, Centaurea stoebe L. is divided into two subspecies: the diploid C. stoebe subsp. stoebe (2n=18; 2x) and the tetraploid C. stoebe subsp. micranthos (Gugler) Hayek (2n=36; 4x). Tetraploids originated from allopolyploidization between diploids and a yet unknown, closely related, second parental species (Mráz et al. 2012a). In their sympatric range, diploids represent the major cytotype and prevail particularly in Central Europe, while tetraploids are more prevalent in South Eastern Europe (Broennimann et al. 2014; Rosche et al. 2016). The cytotypes have a similar morphology, but differ in their life cycle: diploids are short-lived and monocarpic, whereas tetraploids are perennial and polycarpic (Ochsmann 2000; Mráz et al. 2011). The increased longevity of tetraploids has been interpreted as an improved ability to colonize disturbed environments in comparison to diploids (Mráz et al. 2012b; Rosche et al. 2016, 2018). EU4x are thought to be pre-adapted to the drier summers in western North America than EU2x (Treier et al. 2009), because of characteristics like increased leaf dry matter content, reduced specific leaf area and faster root growth (Henery et al. 2010; Mráz et al. 2011; Collins et al. 2013). In contrast, other research found that drought tolerance in C. stoebe is more related to among-population variation within cytotypes than any general differences between the cytotypes (Collins et al. 2011; Mráz et al. 2014).

At the end of the 19th century, both cytotypes were likely introduced to North America, but only tetraploids established (Treier et al. 2009; Sun et al. 2016) and became a highly harmful invader causing immense ecological and economic damage

(Müller-Schärer 1991; van Driesche et al. 2002; Maron and Marler 2008). Their range expansion appeared to stimulate post-introduction evolutionary changes between EU4x and NA4x. Compared to EU4x, NA4x was shown to flower earlier (Hahn and Müller-Schärer 2013), have a more pronounced polycarpic life cycle (Henery et al. 2010), a higher competitive ability (Ridenour et al. 2008) and an increased population growth rate (Hahn et al. 2012). The increased population growth rate was related to an increased seed production (Hahn et al. 2012) and a higher seed-ling emergence (Hahn et al. 2013). In contrast to these findings supporting post-introduction evolution, phenotypic plasticity was similar between EU4x and NA4x (Hahn et al. 2012), and also leaf traits related to drought tolerance did not differ between but rather within EU4x and NA4x (Henery et al. 2010; Mráz et al. 2014).

Sampling

We sampled seeds from 208 populations (83 EU2x, 96 EU4x and 29 NA4x, Fig. 1, Suppl. material 1: table S1), covering broad spatial-environmental gradients in the sympatric native ranges of both cytotypes and also from both the western and eastern invasion routes of NA4x (sensu Broennimann et al. 2014). We collected



Figure 1. Overview of the sampled *Centaurea stoebe* populations across the three geo-cytotypes. The panels **A**, **B** show the geographical distribution of the populations across the native, European and the non-native, North American ranges, respectively. Circles represent populations from (semi-)natural habitats, whereas squares represent populations from ruderal habitats. In panel **C** populations are plotted according to their mean annual temperature and precipitation on a Whittaker diagram showing the classification of the main terrestrial biomes. Panel **D** shows the comparison of climatic water deficit among the three geo-cytotypes. Geo-cytotypes: native diploids (EU2x) = blue, native tetraploids (EU4x) = purple and non-native tetraploids (NA4x) = red. Climatic water deficit data was downloaded from the TerraClimate database (Abatzo-glou et al. 2018). Annual precipitation and annual mean temperature data were downloaded from the CliMond database (Kriticos et al. 2012).

ripe capitula from < 20 individuals (seed families) distributed throughout every population with a minimum distance of 2 m between sampled individuals. To assess the cytotype of all populations, we pooled samples per population (one seed per seed family) and used flow cytometry following the same protocol as in Mráz et al. (2011). Seeds were stored at room temperature until the germination experiment was initiated in each autumn in the respective year of each sampling. Suppl. material 1: table S1 provides details on the sampling year and the number of seed families sampled within each population.

For each population, we distinguished between ruderal and (semi-)natural habitats following the methodology in Broennimann et al. (2014). Industrial and agricultural habitats were considered ruderal, whereas populations from rock outcrops, natural and semi-natural grasslands, natural and diluvial sediments were considered (semi-)natural. We sampled 46 (semi-)natural and 37 ruderal EU2x populations, 34 (semi-)natural and 62 ruderal EU4x populations and 11 (semi-)natural and 18 ruderal NA4x populations (Suppl. material 1: table S1). Population size was assessed by estimating the number of flowering individuals. To determine the local aridity patterns of sampled populations, we obtained seasonal data (1958–2022) on the climatic water deficit (CWD) from the TerraClimate dataset (Abatzoglou et al. 2018) at a spatial resolution of ca. 4 km². The CWD is calculated as the difference between the actual and potential evapotranspiration. From the obtained seasonal data, we calculated the annual mean CWD of each sampled population (Nagy et al. 2024).

Germination trials

We used only undamaged, healthy-looking seeds (e.g., black/brown coloured and well rounded). For each seed-family, 20 seeds were weighed using a fine scale. Ten of these 20 seeds were placed on a filter paper moistened with tap water in a 10–cm Petri dish (no replication of seed families within each treatment). Seeds were germinated in a growth chamber at 20:10 °C with a 12:12 h photoperiod; positions of Petri dishes were randomized daily. Germination was monitored daily over 14 days. Prolonging this germination trial may have resulted in further germination. However, previous studies on *C. stoebe* showed that the vast majority of seeds germinated by the end of this period (Hahn et al. 2013; Rosche et al. 2017). A seed was considered germinated once the radicle emerged and was removed from the Petri dish immediately after germination.

The remaining ten seeds per seed family were germinated under experimental drought. To simulate drought stress, we used D-Mannitol solution in place of water to moisten the filter paper and sealed Petri dishes with Parafilm[®]. Mannitol decreases osmotic pressure and therefore reduces the water availability, and is more stable than other osmotic agents such as polyethylene glycol (Thill et al. 1979). In order to identify a suitable drought treatment that would inhibit but not prevent germination, we randomly selected 97 seed families evenly distributed among the GCTs and germinated them for 14 days in tap water and in three different D-Mannitol solutions of -0.25 MPa (0.1 mol/l), -0.5 MPa (0.2 mol/l) and -1 MPa (0.4 mol/l). This preliminary experiment revealed that there was a significant difference only between the water and the -1 MPa treatment (F = 27.09, p < 0.001; Suppl. material 1: fig. S1). Thus, we applied two treatments in which the Petri dishes were either filled with tap water (hereafter referred to as 'mesic') or with a D-Mannitol solution of -1 MPa (hereafter referred to as 'dry').

Early seedling traits

To assess early seedling traits, we used the first seed per seed family that germinated from the mesic treatment. For each seed family, we transferred this seedling into a new Petri dish in the growth chamber (set up as described previously). At this point, we measured radicle length with ImageJ (Schneider et al. 2012). We then monitored the seedlings until the first sign of the first true leaf appeared. This developmental stage initiates the transition from auto- to heterotrophy (Harper 1977). We recorded the number of days from germination until the day of first true leaf appearance (DTL). We measured the radicle length again for root elongation rate (RER = (length at day of germination - length at day of first true leaf) / day of first true leaf). We then harvested the seedling, dividing it into above- and below-ground biomass, dried at 60 °C for 48 h, and then calculated the root:shoot ratio (RSR = belowground biomass / aboveground biomass) and the relative growth rate (RGR = (belowground biomass + aboveground biomass) / day of first true leaf).

Statistical analysis

All statistical analyses were performed with R version 4.2.3 (R Core Team 2023). To assess germination probability through time, we used time-to-event analyses which are considered a powerful method for analysing germination data and meet more statistical assumptions than e.g., non-linear regression models (McNair et al. 2012; Romano and Stevanato 2020; Onofri et al. 2022). The time-to-event analyses based on mixed-effects Cox-regression models (Cox 1979), using the 'coxme' function of the R-package coxme ver. 2.2.18.1 (Therneau 2022a). These models apply Cox proportional hazards to identify significant predictors of the hazard ratio (HR). The HR indicates the probability to experience a germination event at every given point in time over the whole observation period compared to a reference group. Explanatory variables of our models were geo-cytotype (GCT) in interaction with habitat type and GCT in interaction with climatic water deficit (CWD). Our reference groups were: EU4x and (semi-)natural habitat type. We set population nested within year of germination trial as random effects. Furthermore, we included log_-transformed seed mass and log_-transformed population size as covariates to account for potential maternal and Allee effects, respectively. All numerical predictors were centred and scaled. We ran two separate mixed-effects models for both mesic and dry treatments. The statistical assumptions of the Cox proportional hazards models were checked based on Schoenfeld residuals and their graphical assessments (McNair et. al 2012). Moreover, we calculated the variance inflation factor to test for multicolinearity between our variables. We displayed the germination patterns using Kaplan-Meier curves (Kaplan and Meier 1992) with the 'survfit' function of the R-package survival ver. 3.5.3 (Therneau 2022b).

To identify significant predictors of germination success at the end of the 14-day period (binomial, cumulative values over population means), we used generalized linear mixed-effects models. We used the same explanatory variables as for the mixed-effects Cox-regression models, setting population nested within year of germination trial as random effects. Again, we ran two separate models for both mesic and dry treatments.

For the early seedling traits and the seed mass, we used linear mixed-effects models analysing the following traits as response variables: log_e-transformed RGR, log_e-transformed RER, log_e-transformed DTL, untransformed RSR and log_e-trans-

formed seed mass. Explanatory variables and random effects were identical to the generalized mixed-effect models on germination success. In all linear models, we assessed statistical significances of the predictors through stepwise backward model selection. The minimal adequate models were achieved using χ^2 -tests in the R-package *lme4* ver. 1.1.32 (Bates et al. 2015).

Results

Seed mass

Seed mass differed among GCTs ($\chi^2 = 7.4$, p < 0.05). EU4x populations had higher seed mass than EU2x (p < 0.01, Suppl. material 1: table S2, fig. S2A) but there was no difference between NA4x and either native GCT (EU4x, EU2x). Moreover, seed mass was negatively correlated with CWD (p < 0.01, Suppl. material 1: fig. S2B).

Germination probability

We monitored germination probability – the chance of a germination event happening on successive days - across a total of 23,928 seeds (9,608 EU2x, 10,713 EU4x, 3,607 NA4x) from 208 C. stoebe populations. In the mesic treatment, germination patterns differed among geo-cytotypes (GCTs: $\chi^2 = 51.4$, p < 0.001, Fig. 2) with NA4x having an 86% greater germination probability compared to EU4x (HR = 1.86, p < 0.001, Fig. 2, Suppl. material 1: table S3) and EU2x having a 21% lower germination probability compared to EU4x (HR = 0.79, p < 0.01). However, the difference between the native cytotypes only occurred with seeds from (semi-)natural habitats (p < 0.001) but not with those from ruderal habitats (p = 0.68). Habitat type as a main effect also significantly affected germination probability ($\chi^2 = 33.8$, *p* < 0.001): overall, seeds from ruderal habitats showed 45% higher germination probability than those from (semi-)natural habitats (HR = 1.45). Furthermore, the Cox-regression model revealed that increasing seed mass affected germination probability positively (HR = 1.26, χ^2 = 49.5, *p* < 0.001) while increasing climatic water deficit (CWD) had a negative effect (HR = 0.85, χ^2 = 8.7, *p* < 0.01) There was also an interaction between GCT and CWD (χ^2 = 24.6, *p* < 0.001), indicating that the relationship between germination probability and CWD was most pronounced in EU2x.

In the dry treatment, the germination probability of all GCTs declined (Fig. 2) and there was no difference among GCTs anymore ($\chi^2 = 3.4$, p = 0.18). However, germination was again affected by habitat type ($\chi^2 = 9.1$, p < 0.01): seeds from ruderal habitats had a 44% increased germination probability compared to seeds from (semi-)natural habitats (HR = 1.44). Still, seed mass affected germination probability positively ($\chi^2 = 36.9$, p < 0.001) but to a higher extent than in the mesic treatment (HR = 1.64). In contrast to the mesic treatment, the germination probability was no longer related to CWD ($\chi^2 = 0.1$, p = 0.81). However, population size affected germination probability positively (HR = 1.19, $\chi^2 = 6.8$, p < 0.01).

Total germination

In the mesic treatment, total germination differed among GCTs ($\chi^2 = 405.0$, p < 0.001; Fig. 3, Suppl. material 1: table S4) with NA4x seeds showing higher total germination than EU4x, whereas EU2x had lower total germination than



Figure 2. Kaplan-Meier curves of germination probability and their 95% confidence intervals for the three geo-cytotypes of *Centaurea stoebe* in (semi-)natural (left panels) and ruderal (right panels) habitats. Kaplan-Meier curves are presented for the mesic (upper panels) and the dry treatment (lower panels). Geo-cytotypes: native-range diploids (EU2x) = blue, native-range tetraploids (EU4x) = purple and non-native range tetraploids (NA4x) = red. The dotted lines represent the day at which 50% of the seeds that did germinate had germinated.

EU4x. There was also a difference between habitat types ($\chi^2 = 57.0$, p < 0.001). Populations from ruderal habitats had higher total germination success than those from (semi-)natural habitats. Moreover, CWD was negatively correlated with total germination ($\chi^2 = 308.2$, p < 0.001) and there was a significant interaction between GCT and CWD ($\chi^2 = 11.9$, p < 0.01), with the effect of CWD on germination more pronounced for EU2x than for the other two GCTs. Seed mass ($\chi^2 = 138.0$, p < 0.001) had a positive effect on total germination while population size had no effect ($\chi^2 = 0.53$, p = 0.47).

In the dry treatment, total germination again differed among GCTs ($\chi^2 = 20.8$, p < 0.001), but in contrast to the mesic treatment, total germination was comparable between EU4x and NA4x, whereas EU2x showed greater total germination than EU4x. Again, total germination differed between habitat types ($\chi^2 = 33.8$, p < 0.001) with populations from ruderal habitats showing greater germination success. Contrary to the mesic treatment, CWD was positively correlated with total germination ($\chi^2 = 5.8$, p < 0.05) and the effect of CWD differed among GCTs ($\chi^2 = 29.9$, p < 0.001), with a positive relationship evident only for NA4x. Moreover, seed mass ($\chi^2 = 102.4$, p < 0.001) and population size ($\chi^2 = 9.9$, p < 0.01) were positively correlated with total germination under dry conditions.

Early seedling traits

There was no difference in any of the early seedling traits between EU2x and EU4x (Suppl. material 1: table S5). In contrast, NA4x developed their first true leaf earlier than both native GCTs ($\chi^2 = 13.29$, p < 0.01, Fig. 4A) and had a higher RGR ($\chi^2 = 13.89$, p < 0.001, Fig. 4B). Early seedling traits did not differ between habitat types except for RSR. Ruderal populations had higher RSR than (semi-)natural ones, regardless of GCT, ($\chi^2 = 4.79$, p < 0.05, Suppl. material 1: fig. S4). Seed mass had a significant effect on some early seedling traits investigated (Suppl. material 1: table S5, fig. S4).



Figure 3. Relationship between climatic water deficit and total germination across 208 populations from the three geo-cytotypes of *Centaurea stoebe* in the mesic treatment (upper panel) and the dry treatment (lower panel). Geo-cytotypes: native diploids (EU2x) = blue, native tetraploids (EU4x) = purple and non-native tetraploids (NA4x) = red.



Figure 4. Differences among the three geo-cytoypes of *Centaurea stoebe* in early seedling traits. The panels **A**, **B** show the days needed to develop the first true leaf and the relative growth rate, respectively. Geo-cytotypes: native diploids (EU2x) = blue, native tetraploids (EU4x) = purple and non-native tetraploids (NA4x) = red. For model estimates see Suppl. material 1: table S5.

Discussion

Pre-adaptive differences between EU4x and EU2x

Under mesic conditions, EU4x seeds showed a higher germination probability through time and a greater total germination than seeds of EU2x. These findings contrast with previous results from Hahn et al. (2013) who did not find such differences between the cytotypes. We studied more populations covering broader environmental gradients than Hahn et al. (2013) and we used Cox-regression models instead of the linear models in Hahn et al. (2013). However, our results are consistent with other literature reporting that polyploidy can be associated with higher germination rates (e.g., Stevens et al. 2020; Selvi and Vivona 2021). This superior germination performance of polyploids has been linked to higher seed mass (Bretagnolle et al. 1995; Stevens et al. 2020). We indeed found that EU4x had heavier seeds than EU2x, and there was a strong, positive correlation between germination probability and seed mass, independent of GCT. Given the strong effects of germination success on population growth in founder populations of *C. stoebe* (Hahn et al. 2012), the superior germination performance of EU4x may display an important pre-adaptive advantage to colonize novel ranges compared with EU2x.

Under simulated drought, the superior germination performance of tetraploids disappeared. Instead, EU2x showed greater total germination than EU4x under dry conditions. Polyploids often shift their germination behaviour under unfavourable conditions (Hoya et al. 2007; Stevens et al. 2020; Rutland et al. 2021) and the seeds of tetraploid *C. stoebe* can remain viable in the seed bank for at least eight years (Davis et al. 1993). Delaying germination until better conditions may be thus a beneficial strategy for tetraploids colonizing non-native ranges with novel environmental conditions. Moreover, the positive effect of seed mass on germination probability almost tripled in the dry treatment, suggesting that seed size is more important under harsh conditions, which is consistent with evidence that heavier seeds cope better with environmental stress (Milberg and Lamont 1997; Paz and Martínez-Ramos 2003; Hallett et al. 2011; but see Yi et al. 2019).

Given the differences in germination probabilities and seed masses between the cytotypes, it is surprising that EU2x did not differ from EU4x in early seedling traits. Larger seeds are expected to result in faster seedling growth, especially in root mass allocation and seedling size (Bretagnolle et al. 1995; Kidson and Westoby 2000; Hallett et al. 2011; but see Norden et al. 2009; Tumpa et al. 2021). A previous study found that EU4x seeds performed better in all seedling traits they studied (Collins et al. 2013). However, they used five populations per cytotype and terminated their experiment after 16 days rather than in response to the developmental stage of seedlings as we did. A recent review demonstrated that such methodological differences can substantially affect the results of seedling traits studies (Winkler et al. 2024).

Post-introduction evolution between EU4x and NA4x

NA4x showed greater germination than EU4x, which contrasts with Hahn et al. (2013) who did not find such differences. Again, the contrasting results may be explained by the different study designs and analyses. In the dry treatment, however, the superior germination of NA4x over EU4x disappeared. This is surprising given the niche shift towards a drier climate tetraploids experienced during their

range expansion in North America (Broennimann et al. 2007). However, it may be possible that the novel conditions in the non-native range were selected for more conservative germination behaviour under drought.

Regarding early seedling traits, we found seedlings of NA4x to develop their first true leaf faster than those of EU4x while also accumulating more overall biomass in this shorter time period. This faster development and seedling growth is not only important because it marks the transition from auto- to heterotrophy (Harper 1977), but it may also increase seedling drought resistance (Larson et al. 2020) and might be advantageous regarding competitive interactions with resident species in the invaded range (Gioria et al. 2016). Indeed, NA4x seedlings were recently found to establish faster than resident species in the invaded plant communities in western USA (Slate et al. unpublished data). Considering the cascading effects of seedling performance on later life stages (Donohue et al. 2010; Gioria and Pyšek 2017), the faster seedling growth may also partly explain the common observation of increased adult plant performance of NA4x compared to EU4x populations under diverse common garden conditions (e.g., Blumenthal and Hufbauer 2007; Ridenour et al. 2008; He et al. 2010; Hahn and Müller-Schärer 2013). Such post-introduction evolution towards high performance genotypes in the non-native range is well-known for adult plant performance traits in numerous invaders (Parker et al. 2013) and could be a response to novel biotic and abiotic interactions in the non-native range (Bates and Bertelsmeier 2021; Sheng et al. 2022). However, so far this phenomenon could not be shown for the recruitment life stage. More experiments are needed to understand the eco-evolutionary processes selecting at this early life stage and if so, how this may affect adult plant growth.

Among-population variation within the three geo-cytotypes

The negative correlation between the climatic water deficit and germination probability could be due to reduced energy content of the seeds from dry populations, where mother plants likely faced drought stress during seed production (Hampton et al. 2013; Heinicke et al. 2016). However, in the dry treatment, total germination was positively affected by increasing climatic water deficit. Local adaptation in populations from drier regions may increase likelihood of germination under dry conditions (Godfree et al. 2017; Veselá et al. 2021; Gya et al. 2023). Seedling performance traits were not affected by climatic water deficit, although seedlings sampled from dry environments are assumed to show a more conservative growth, e.g., investing more into root than shoot biomass allocation (Larson et al. 2020; Gya et al. 2023). Because we did not investigate seedling performance traits in the dry treatment, our results rather explore the population level variability across CWD while future research is needed to understand seedling growth under experimental drought (Dhanda et al. 2004; Birkeli et al. 2023; Winkler et al. 2024).

The higher germination in ruderal than (semi-)natural habitats, indicates a selection toward genotypes that quickly occupy open niches in ruderal habitats whereas in (semi-)natural habitats there may be a selection for genotypes that show more conservative germination behaviour (Hufbauer et al. 2012). The higher root:shoot ratio in ruderal than (semi-)natural habitats may indicate adaptation to fluctuating water availability (Chapin et al. 1993). Ruderal habitats usually have less vegetation cover and tend to have greater evapotranspiration than (semi-)natural habitats (Bai et al. 2020). The significant differences we found in recruitment traits between habitat types correspond to pronounced differences between ruderal vs. (semi-)natural *C. stoebe* populations in their adult plant growth performance (Rosche et al. 2018).

Our results support current concepts emphasizing that populations within native and non-native ranges (Colautti and Lau 2015) and within cytotypes (Rosche et al. 2018) may vary strongly in their population history. Such among-population variation within GCTs may be particularly pronounced for recruitment traits that are under strong natural selection (Baskin and Baskin 1998; Donohue et al. 2010; Cochrane et al. 2015). This means that the choice of populations can affect the outcome of experiments, particularly if habitat types and local aridity regimes were not sampled to similar extents among GCTs (Rosche et al. 2018; 2019). Moreover, with a view to ecological restoration, we strongly recommend choosing seed sources that correspond to the target habitat type to avoid maladapted germination and seedling growth behavior (Larson et al. 2020).

Conclusions

Our study is the first in the recruitment life stage to show that a biological invasion can be associated with a combination of pre-adaptive differences between cytotypes and post-introduction evolution of native and non-native populations. Because early seedling establishment is a crucial bottleneck in the life-cycle of plants and has cascading effects on all later individual performances, we call for further eco-evolutionary research at this life stage. Given that we recorded early seedling traits only under mesic conditions, we recommend that future research should investigate those traits also at suboptimal conditions. Furthermore, we emphasize that adaptive differentiation can be even more pronounced within than among GCTs. To avoid misleading conclusions from rather simplistic native vs. non-native or diploid vs. polyploid comparisons, future experimental studies should consider that not only the habitat type but also environmental gradients should be sampled to a broad and comparable extent between ranges and cytotypes.

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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

C.R. and K.K. designed the study. K.K. wrote the manuscript with the help of C.R.. K.K. analyzed the data with the help of M.H. and C.R.. All authors contributed to the sampling, discussed the results and contributed to the final manuscript.

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Data availability

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

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Supplementary material 1

Supplementary information

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Data type: docx

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Supplementary material 2

Raw data

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Data type: xlsx

Explanation note: Experimental data.

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