

Regional models do not outperform continental models for invasive species

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Abstract

Aim: Species distribution models can guide invasive species prevention and management by characterizing invasion risk across space. However, extrapolation and transferability issues pose challenges for developing useful models for invasive species. Previous work has emphasized the importance of including all available occurrences in model estimation, but managers attuned to local processes may be skeptical of models based on a broad spatial extent if they suspect the captured responses reflect those of other regions where data are more numerous. We asked whether species distribution models for invasive plants performed better when developed at national versus regional extents.

Location: Continental United States.

Methods: We developed ensembles of species distribution models trained nationally, on sagebrush habitat, or on sagebrush habitat within three ecoregions (Great Basin, eastern sagebrush, and Great Plains) for nine invasive plants of interest for early detection and rapid response at local or regional scales. We compared the performance of national versus regional models using spatially independent withheld test data from each of the three ecoregions.

Results: We found that models trained using a national spatial extent tended to perform better than regionally trained models. Regional models did not outperform national ones even when considerable occurrence data were available for model estimation within the focal region. Information was often unavailable to fit informative regional models precisely in those areas of greatest interest for early detection and rapid response.

Main conclusions: Habitat suitability models for invasive plant species trained at a continental extent can reduce extrapolation while maximizing information on species' responses to environmental variation. Standard modeling methods can capture spatially varying limiting factors, while regional or hierarchical models may only be advantageous when populations differ in their responses to environmental conditions, a condition expected to be relatively rare at the expanding boundaries of invasive species' distributions.

Keywords

Early detection and rapid response, invasion risk, model transferability, species distribution models

Introduction

Organisms' responses to environmental variation underlie patterns of distribution and abundance and are the basis for correlative statistical tools such as species distribution models (SDMs; Franklin 2010). Among the challenges of such models are that 1) the relationships between environmental conditions and organismal response can vary over space and time, and 2) outcomes under new conditions are difficult to predict (Sequeira et al. 2018; Yates et al. 2018). These twin challenges, transferability and extrapolation, can point to opposing solutions when interest is in predicting habitat suitability in a region beyond the core of a species' range (Werkowska et al. 2017; Sequeira et al. 2018). Transferability challenges could favor limiting both estimation and prediction to within the region of interest when such data are available and where responses to environmental conditions are thought to be distinctive (Barbet-Massin et al. 2018). However, while a regional approach may capture key limiting factors, it excludes the full range of environmental conditions under which data are available and hence can lead to unnecessary extrapolation and errors in estimated suitability (e.g., Fitzpatrick et al. 2007; Broennimann and Guisan 2008).

Predicting suitability for invasive species exemplifies challenges with both transferability and extrapolation (Elith et al. 2010). Wherever invasive species are still spreading, correlative models can conflate this lag in time (i.e., lack of equilibrium) with a lack of suitability. A common recommendation is to develop the most inclusive view of invasion risk by estimating models based on both the native and invaded ranges to capture the species' complete environmental associations and minimize extrapolation (Fitzpatrick et al. 2007; Broennimann and Guisan 2008). However, where populations are differentiated in their responses to environmental conditions, surveillance and management may be more effectively guided by locally or regionally tuned approaches because of poor model transferability (e.g., Connor et al. 2019; Collart et al. 2021). Studies focused on native species have found modeling intraspecific subsets of the data based on genetic or regional groupings improved distribution model predictions (Chardon et al. 2020). Further, regional models performed better at predicting distributions within the margins of species ranges, where different environmental predictors were most important (Vale et al. 2014; Connor et al. 2019). Marginal or poorly sampled populations may also contribute little to model estimation if training data are

heavily dominated by a better sampled portion of a species' range (Pearman et al. 2010; Hällfors et al. 2016); conversely, limiting model estimation to sparse data in a subset of the range may lead to low model quality. Given the potential for population differentiation within species' invaded ranges (e.g., Colautti and Barrett 2013), models of invasive species' distributions may face important trade-offs between inclusivity versus regional applicability, as well as practical data limitations in newly invaded areas. Meta-analyses of studies that trained models using native range only, invaded range only or global range did not find that global models perform better than models generated in the range of interest, and indicate that superiority of global model performance could be a statistical artefact because test data are not independent (Liu et al. 2020b).

Early detection and rapid response (**EDRR**) activities aim to prevent establishment, spread, and impact through surveillance and rapid management action, and can minimize invasions in new regions (Reaser et al. 2020). Sagebrush (*Artemisia* sp.) habitats in the western United States (U.S.) provide habitat for many wildlife species and face multiple stressors including invasive species, altered fire regimes, climate change, and energy development (Davies et al. 2011; Coates et al. 2016). Crist et al. (2019) have developed a list of invasive plants that have no, patchy, or limited presence on a state-by-state basis within sagebrush habitat. Their approach emphasized the potential for ongoing spread and geographic differences in invasion status, as species that are well established within one state may still warrant EDRR elsewhere. For these regional 'EDRR species', species distribution models can guide surveillance by identifying areas with high invasion risk (Brooks and Klinger 2009). However, one concern we have heard from within the management community is that models trained with a broad geographic extent could miss regionally and locally relevant limiting factors if important signals were swamped by other portions of the range.

For a set of nine species recognized as EDRR targets within sagebrush habitats (Crist et al. 2019), we characterized each species' relationship to sagebrush communities to understand habitat associations and degree of sage specialization. We then compared the performance of national (here used to refer to the conterminous U.S.) versus regional species distribution models. We compared regional models to national, instead of global, models because of the availability of a wider breadth of predictors within the conterminous U.S., including higher quality data than are available globally (e.g., for soils), and finer spatial resolution of predictors focused on the U.S. compared to global versions. Appropriate methods to account for sampling biases are also likely to differ between a native range, where a species is likely closer to equilibrium, and a novel range, which complicates background selection when pooling records from native and invaded ranges (Elith et al. 2010; Jarnevich et al. 2017). In addition, all species in question have been in the U.S. since at least 1957 (based on earliest occurrence records; GBIF.org 2022), giving them time to potentially develop local adaptations and providing numerous occurrence points for model estimation (Liu et al. 2020a; b). Thus, we fit species distribution models for each species across the U.S., from all sagebrush within the U.S., and separately within sagebrush habitats in each of three ecoregions (Great Basin, eastern sage, Great Plains). Models trained on sagebrush only

were fit to allow for any response curves specific to sagebrush habitats (within which models were also tested). We evaluated model performance using withheld spatially independent validation data within each region. We hypothesized that given sufficient data and variation in environmental responses, a regional model evaluated with test data from within the region could outperform a national model. Our results evaluate whether national models can sufficiently capture invasion risk across ecoregions, or whether estimation of models for each region improves the credibility of the outputs for on-the-ground management.

Methods

Study area

We used a combination of level 2 and 3 EPA ecoregion designations (U.S. Environmental Protection Agency 2013) to create three regional study areas: the Great Basin (regions 10.1.8/3/5), eastern sage (region 6.2), and Great Plains (regions 9.2, 9.3, and 9.4) regions. Within each regional boundary, we further restricted the study area for each region to sagebrush habitat, defined by 30 m² cells of greater than 0% sagebrush presence as designated by the National Land Cover Data set (**NLCD**) shrubland sagebrush rangeland fractional component product (Xian et al. 2015).

We created a spatial split of the occurrence data for model validation, as random splits typically underestimate prediction error (Roberts et al. 2017; Fourcade et al. 2018). Within each of the three regions we designated a central longitudinal test strip that contained 10% of the sagebrush cells within the region (Fig. 1; Suppl. material 1: Table S1). Occurrence data points within sage habitat inside these test strips were withheld from model fitting and used to evaluate model performance. In addition to these three regional model estimation extents, we considered two larger spatial extents: the continental U.S., and all sagebrush habitat within the continental U.S., defined as above based on Xian et al. (2015; hereafter “all sage”).

Study species

We selected nine plants from a list of invasive species for EDRR activities within states of the eastern sage region (Crist et al. 2019) that had at least 50 training occurrence records and 30 test strip records in at least one study region. These included seven forb/herbs as defined by the USDA PLANTS database (USDA NRCS 2019): *Centaurea diffusa* Lam. [diffuse knapweed], *C. solstitialis* L. [yellow star-thistle], *C. virgata* Lam. [squarrose knapweed], *Chondrilla juncea* L. [rush skeletonweed], *Halogeton glomeratus* (M. Bieb.) C.A. Mey. [saltlover, halogeton], *Rhaponticum repens* (L.) Hidalgo Syn *Acroptilon repens* [Russian knapweed] and *Salvia aethiopis* L. [Mediterranean sage]. We also included two winter annual grass species: *Taeniatherum caput-medusae* (L.) Nevski [medusahead rye] and *Ventenata dubia* (Leers) Coss. [ventenata, North Africa grass].

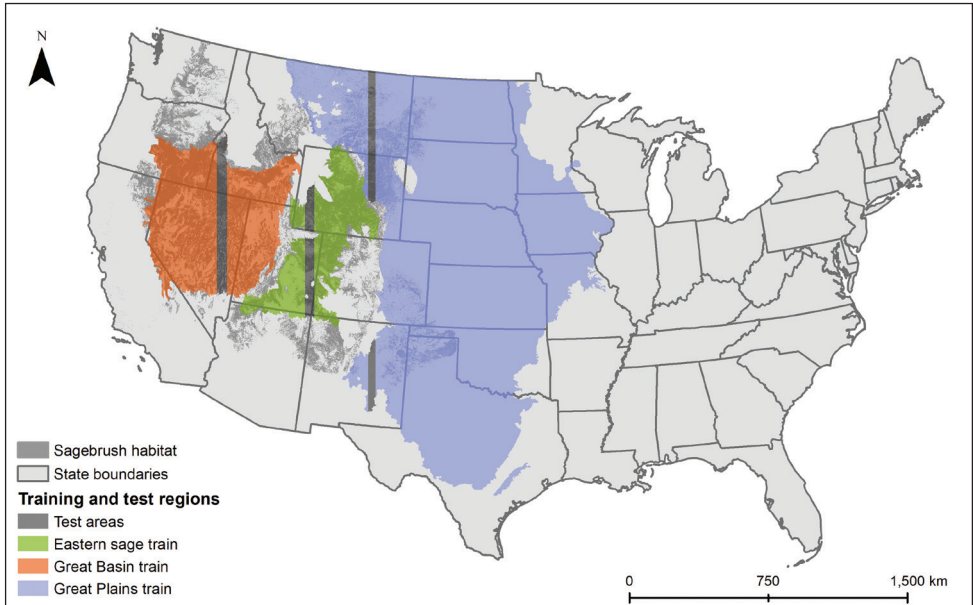


Figure 1. We compared five geographic extents for model estimation while holding validation data constant (occurrence points within dark grey vertical shaded areas). Two geographic training extents were continental and three were regional, and we fit an ensemble of distribution models to the occurrence points for each species within each estimation extent. These extents for model estimation were: 1) the continental United States; 2) all sagebrush habitat within the continental U.S. (gray shading within the western U.S.); 3) sagebrush within eastern sage; 4) sagebrush within the Great Basin; and 5) sagebrush within the Great Plains. Within each of the three regions (shown via colored polygons), we created a test strip (vertical shaded areas) centered on sagebrush habitats, and withheld occurrence points for model performance comparisons. We asked whether a regional or continental training extent yielded higher performance within these test strips, as measured by the Boyce index values.

We aggregated occurrence data from existing data sets following Young et al. (2020), including Global Biodiversity Information Facility (GBIF; GBIF.org 2021), Biodiversity Information Serving Our Nation (BISON; individual data sources and all data used in this study are available as a USGS data release Jarnevich et al. 2022), the Early Detection and Distribution Mapping System (EDDMapS) [all accessed 1 Dec 2020], the Bureau of Land Management’s (BLM) Assessment, Inventory and Management database (obtained June 2020) and the BLM and National Park Service’s National Invasive Species Information Management System (**NISIMS**) (Suppl. material 1: Table S2 [provides more details in an assessment rubric]). All known synonyms and U.S. Department of Agriculture (**USDA**) Plants Database acronyms were collected (excluding subspecies, variants, and hybrids) using the Integrated Taxonomic Information System (**ITIS**; www.itis.gov) as an authoritative taxonomy in the R library ‘taxize’ (Chamberlain and Szocs 2013; Chamberlain et al. 2020). We filtered observations by coordinate uncertainty (≤ 30 m), observation type (observation or

specimen only), and observation date (1980 to present [2020]) to match the time frame of predictors and remove older records which typically have poor geographic accuracy. We removed any records with coordinates corresponding to state or country centroids or other easily identifiable geographic and taxonomic errors. We also checked the entire dataset for duplicate records and confirmed that occurrence locations generally aligned with reported distributions via USDA Plants Database (USDA NRCS 2019). We followed these same methods to obtain location data for species identified as non-native by USDA Plants Database to use as background points to control for sampling biases, as described below. We required 50 occurrence records in a study area to fit a model, and 30 records in a test strip to evaluate models for that strip (Suppl. material 1: Table S3, Fig. S1).

Predictors

We began with a national library of 49 predictors representing climate (water deficit, actual evapotranspiration, precipitation, and temperature average from available years 1981–2018 [see Suppl. material 1: Table S4]), human disturbances, soils, water presence / recurrence, fire history, and land cover created by Young et al. (2020) using the Albers equal area projection with a 90 m² resolution and modified by Engelstad et al. (2022) (Suppl. material 1: Table S4). This list includes predictors thought to be important for determining the distribution of different types of plant species within the continental U.S. For this analysis, we developed a ranking of predictors *a priori* to guide predictor selection for each species based on its natural history, such as winter annual species which use overwinter and spring moisture. We first grouped predictors into ten broad categories and ranked those categories based on our experience developing models for > 140 invasive plants in the continental U.S. (Young et al. 2020; Engelstad et al. 2022) and what environmental characteristics are important for different plant life forms in general. Next, we ranked the predictors within each of these broad categories for each species based on natural history knowledge of each individual species. Beginning with the highest ranked category (ETo), the highest ranked predictor was selected. Then, in the second ranked category, the highest predictor was selected that was not correlated with the first selected predictor for the top ranked category (maximum correlation coefficient of Pearson, Spearman, or Kendall was > 0.7 (Dormann et al. 2013)). An exception was made such that if one category of predictors was eliminated entirely, the second ranked predictor in category 1 would be retained if the highest was correlated with all of category 2 predictors whereas the 2nd ranked allowed for inclusion of another predictor category. Thus, correlation coefficients among predictors were used to limit co-linearity of selected predictors, but correlations with the response variable were not considered in variable selection. We ensured the ratio of presence points to predictors was at least 10:1 (Hosmer and Lemeshow 2000). This resulted in 47 predictors used across all models (Suppl. material 1: Table S4), with a range of 8 to 29 predictors per model.

Analyses

We evaluated the degree to which each species was disproportionately found within sagebrush and within different land cover types by overlaying occurrence points with land cover data. We identified where each focal species has invaded sagebrush communities by overlaying the compiled occurrence data with the NLCD shrubland sagebrush range-land fractional component product (Xian et al. 2015; U.S. Geological Survey and Rigge 2019), defining sagebrush as any location with a > 0 cover value. We also counted the number of presence points for each species within the broad 2016 National Land Cover Data classes (i.e., agricultural, developed, forest, grassland, shrubland). We then calculated the proportion of the total species points found in each class. Because sampling effort can distort distributional assessments (e.g., Sofaer and Jarnevich 2017), we controlled for sampling effort across land cover categories by adjusting observed focal species associations by the habitat-specific number of records for other non-native plants of the same life form (i.e., forb/ herb or graminoid). We plotted the results to assess the degree to which each species disproportionately occurred in sagebrush habitats and each land cover class to better understand habitat preferences and the degree to which different species were sage specialists. These results were interpreted visually, while the target-background method described below similarly accounted for sampling biases within models.

We developed an ensemble of species distribution models for each species and training extent combination containing at least 50 presence locations (Suppl. material 1: Table S3). We fit models using the VisTrails Software for Assisted Habitat Modeling v2.2.0 (SAHM; Morissette et al. 2013) following the methods of Young et al. (2020) and high performance computing (Falgout and Gordon 2021). We implemented five model algorithms [boosted regression tree (Elith et al. 2008), generalized linear model (McCullagh and Nelder 1989), multivariate adaptive regression spline (Elith and Leathwick 2007), Maxent (Phillips et al. 2017), and random forest (Breiman 2001)] and two background point generation methods. One method was a kernel density estimate (KDE) around presence points to weight random background point generation (Elith et al. 2010). The other was target background (Phillips et al. 2009) based on 10,000 randomly selected locations of other non-native species locations within the same broad life form assigned by USDA Plants Database [forb/herb or graminoid] from within a 99% kernel density estimate isopleth (an isopleth is a line representing a constant value, as in a contour line on a topographical map) around the presence points or the full set of life form points if $< 10,000$ points fell within the 99% KDE. KDE isopleths are commonly used to define species' ranges by drawing a polygon to encompass locations (in this case, 99% of them) (Worton 1989) and recommended for range shifting invasive species (Elith et al. 2010). We withheld presence and background locations falling within test strips from estimation of all models. We fit each model using SAHM default parameters for algorithms with 10-fold cross-validation. We examined the difference between train and mean cross-validation values from the area under the receiver operating characteristic curve (AUC) and visually examined response curves to determine if models appeared overfit. In cases where models were

deemed overfit ($\text{trainAUC} - \text{testAUC} > 0.05$ or overly complex response curves), we adjusted model-specific tuning parameters, making the changes that most decreased overfitting while maintaining good cross-validation performance.

Because we only had presence locations, the outputs of the SDM algorithms are interpreted as relative habitat suitability values rather than probabilities. To create an ensemble across algorithms and background methods (10 models) we used of the 10th percentile training presence threshold for each model to produce binary outputs of suitable/unsuitable habitat that we could then sum across the ten models for each species/extent combination. The 10th percentile threshold is calculated for presence-only data based on the omission rate, where the 10% of occurrences with lowest predicted suitability are assumed to occur in poor habitat to avoid over-prediction due to errors or outliers in training locations.

We compared variable importance between regional and national models. We calculated variable importance by permutating values for each predictor across presence and background locations and calculating the difference between the original and permuted AUC values. Within each model, variables were ranked by permutation importance, with the most important variable being the one for which permuting its values led to the greatest decrease in AUC. For the ensemble we averaged the importance across the contributing models.

Because AUC is problematic for presence-background data (Lobo et al. 2008; Peterson et al. 2008; Sofaer et al. 2019a; Jiménez and Soberón 2020), we used the Boyce index to evaluate model performance based on the test data (Hirzel et al. 2006). The Boyce index assesses how much model predictions differ from random expectations by comparing the proportion of occurrences across classes of predicted suitability to the proportion of grid cells in each class. The Boyce index is based on the null expectation that the proportion of validation points expected within a given class is the proportion of the landscape area within that class (i.e., in contrast to sensitivity, which is based only on true positives, it would penalize a model for predicting high suitability everywhere). We calculated the index using the ensemble value (the number of models predicting suitable habitat) as the class bin for the Boyce index, generating 11 classes corresponding to the ensemble values of 0 to 10. Moving from low (zero models predicting suitability) to high (all 10 models predicting suitability), a high performing model will have a higher density of validation points at high ensemble values. Thus, the Boyce index is the Spearman rank correlation between the ordered classes (0–10 in our case) and the proportion of validation points in the focal class divided by the proportion of area in that class. We restricted validation points (Suppl. material 1: Table S3) and area calculations to sagebrush areas within each test strip (Fig. 1). We compared the Boyce index between national and regional training extents for each species, such that each model ensemble was tested on the same set of points for a given species and region.

We also compared the area within our three focal regions predicted to be suitable by each model ensemble. To do this, we turned the ensemble maps into binary suitable/unsuitable maps by classifying any pixel within the region with an ensemble value of 6 or greater as suitable. We then counted the number of suitable pixels anywhere within each of the three different regions for each model ensemble.

Data accessibility statement

The data underpinning the analysis reported in this paper are available by a U.S. Geological Survey data release through the Science Base Repository at <https://doi.org/10.5066/P90AL0PN>.

Results

Most of our focal invasive plants had higher proportions of occurrences in sagebrush habitats compared to occurrences of all invasive plants of the same life form, pointing towards preference for sagebrush habitats after accounting for potential variation in sampling intensity with habitat type. *Ventenata dubia* occurred in sagebrush habitats in a greater proportion relative to occurrence points of other graminoid invasive species, as did *T. caput-medusae* to a lesser extent (Suppl. material 1: Fig. S2). Of the forb/herb species, *C. virgata*, *C. juncea*, and *S. aethiopis* also had positive ratios for sagebrush, indicating that these five species are disproportionately problematic within sagebrush habitats, even after considering sampling biases in occurrence locations. The three *Centaurea* species, *C. juncea*, and *H. glomeratus* all had positive ratios for the eastern sage region compared to other invasive forb species. All species had a positive association with shrubland, which includes sagebrush dominated locations, except *C. juncea* which had a positive association with the herbaceous land cover classes (Suppl. material 1: Fig. S3). It did, however, still have a positive ratio of occurrences in sagebrush everywhere but the Great Plains region (Suppl. material 1: Fig. S2).

Only two species, *C. diffusa* and *R. repens*, had enough locations in all three regions to fit models to all model estimation extents (Suppl. material 1: Table S3, Fig. S1). Patterns in predictions between the different training extents varied by species. *R. repens* mapped predictions varied with the training extent (Fig. 2), but the total area within each region predicted to be suitable by each model ensemble varied less for *R. repens* than for *C. diffusa* (*R. repens* points were closer to 1:1 line in Fig. 3b; Suppl. material 1: Fig. S4a, g). *Centaurea diffusa* model ensembles that were trained on occurrences in sage showed poor extrapolation to other habitats in that they were less restricted to sage compared to the national model ensemble (i.e., sage only models, represented by red triangles in Fig. 3b, fell above the 1:1 line in Fig. 3b); interestingly, several of the national model ensembles for this species predicted less suitable area than their regional counterparts. Variable patterns could be seen for other species, with no clear visual differences in the geographic extent of predicted suitability among models trained on different regions (Suppl. material 1: Fig. S4). Some regional models predicted a lot of suitable habitat outside their training region, potentially extrapolating incorrectly (Suppl. material 1: Fig. S4); models extrapolated to other regions could show higher or lower suitable area than continental models, with extrapolation leading to more variability than interpolation (i.e., the points farthest from the 1:1 line in Fig. 3b are small, indicating they arose via extrapolation). Important predictors between training extents were relatively similar (Suppl. material 1: Fig. S5).

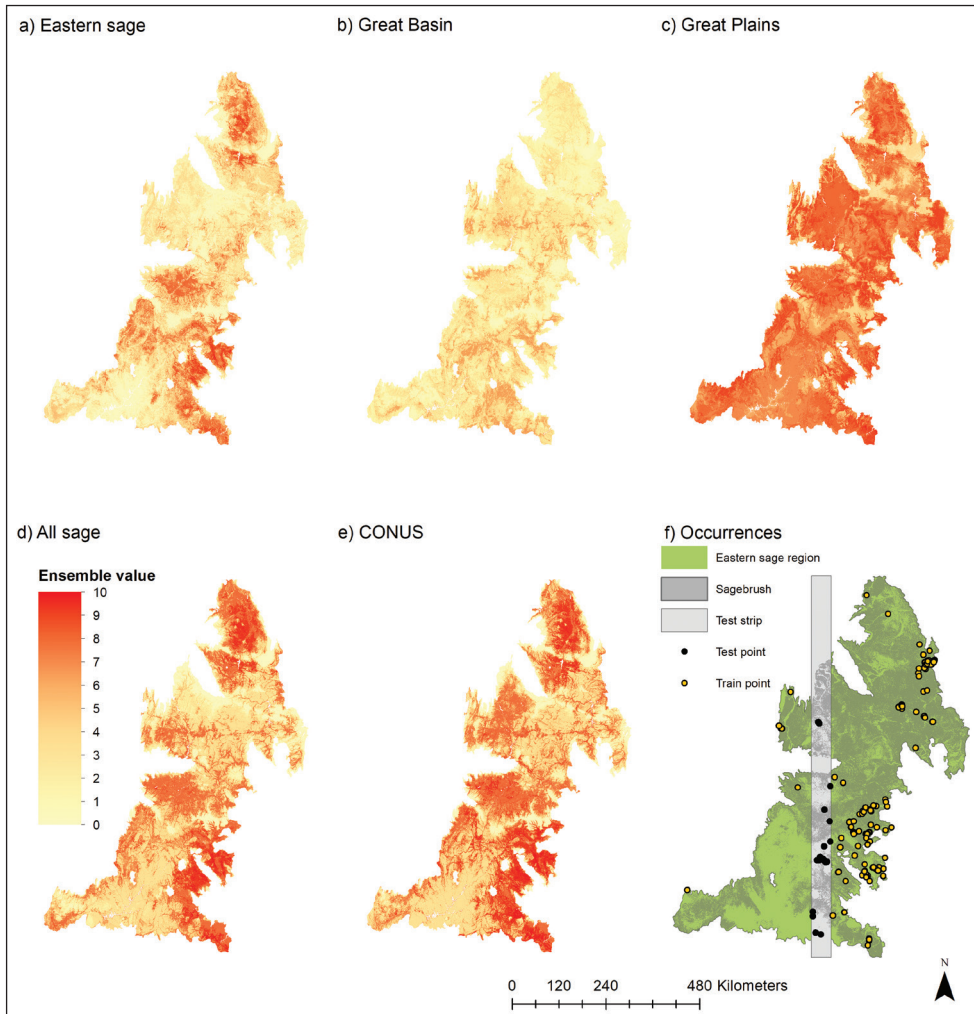


Figure 2. Predicted suitability for *Rhaponticum repens* within the eastern sage region (green region in Fig. 1) from models trained using data from the **A** Eastern sage **B** Great Basin **C** Great Plains **D** All sage, and **E** the continental U.S. (CONUS) (training regions shown in Fig. 1). Maps (**A–E**) show ensembled model predictions, defined as the number of models predicting suitable habitat; **F** shows training and test data for *Rhaponticum repens* within Eastern sage; test data were withheld from estimation of all models and used to create consistent performance assessment sets for each species and region.

Models tested on the region where they were trained were not better than continental U.S. models (paired t-test p-value = 0.07, mean difference = -0.14, i.e., continental models marginally better). Continental U.S. models outperformed models trained on the test region in seven of ten cases (Fig. 3). We had 11 regional test datasets across the nine species which met our criterion of 30 test points within the withheld spatial strip (Suppl. material 1: Table S3). Of these, the continental models or all sagebrush models were better than regional models (including those trained in other

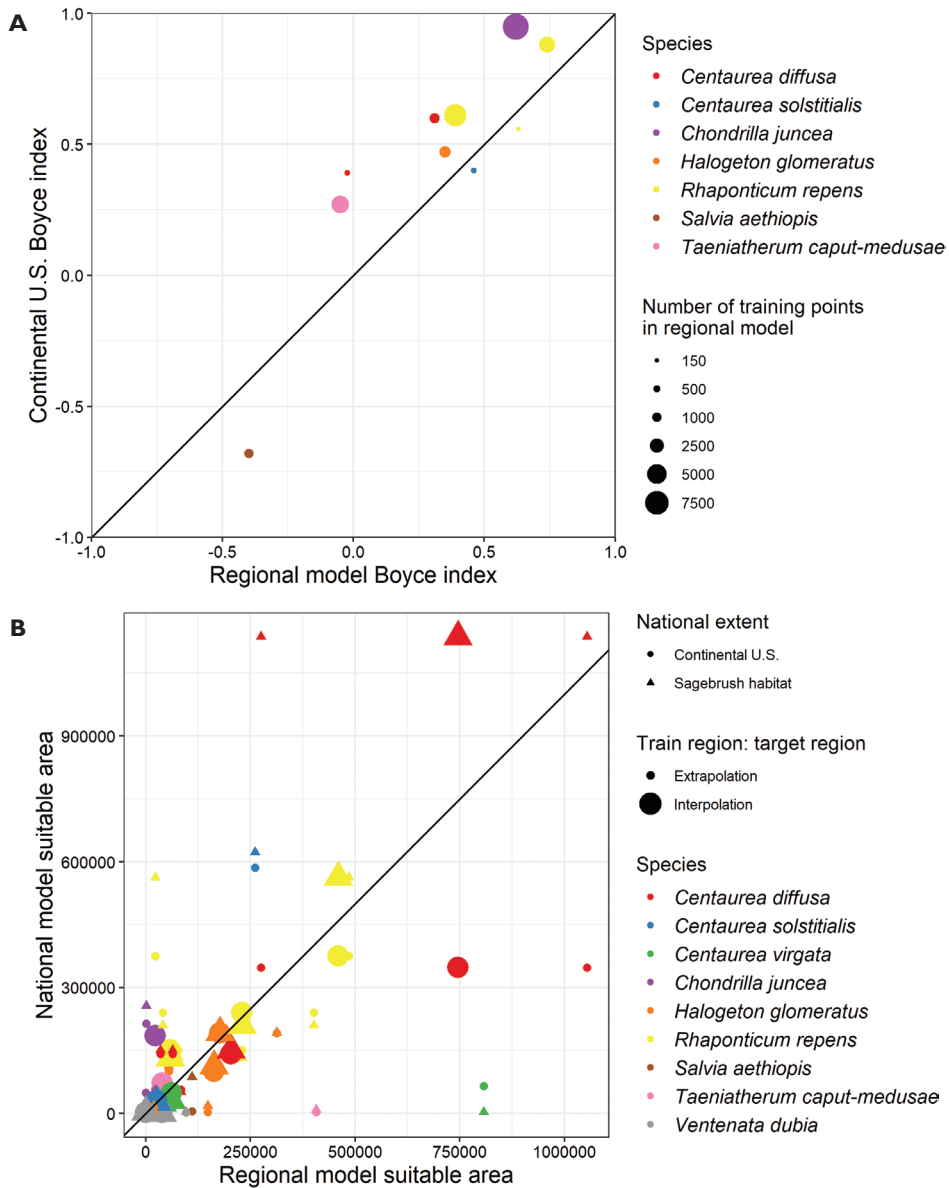


Figure 3. A Regional models did not outperform continental-scale models, even when many points were available within the training region. Boyce index values were calculated for the training region's test strip for both the matching region model ensemble (x-axis) and continental United States model ensemble (y-axis) for each species (color). Species without sufficient occurrence points within the test strip were excluded. Values above the 1-1 line indicate continental U.S. model had better performance; for most species and regions, models with a continental extent performed better even when the number of regional training points was high (i.e., points are above the 1:1 line, even for big points). **B** suitable area predicted by national models (either entire continental U.S. or sagebrush habitat within the U.S.) compared to regional models, where larger size indicated if the focal region considered for area calculation was the same (interpolation) or different (extrapolation) from the regional modeling training region. Values above 1-1 line indicate the national model predicted more suitable habitat.

regions) in most cases (Fig. 3, Suppl. material 1: Fig. S6). A regional model performed better than any other in five cases, of which three were actually models trained in other regions and extrapolated to the test region. The two models trained on the same region as the test strip had a lower Boyce index than models trained on either all sage or other regions. All models for *S. aethiopsis* were poor, with all Boyce index values well below zero, despite decent performance according to typical assessment metrics based on the training data (cross validation AUC > 0.75, with an average per region > 0.89).

While *V. dubia* had enough locations to meet our criteria to develop models for the Great Plains region ($n = 4,246$), the occurrences were all within a relatively small geographic extent, and there were not enough locations for validation (Suppl. material 1: Table S3, Fig. S1). This small geographic extent was problematic in fitting models, where we were unable to obtain enough target background locations within the area around the general extent of occurrences within the region. Three of the five KDE models had poor fit (e.g., training AUC = 0.67 (GLM), 0.695 (MARS), 0.64 (RF)) that we were unable to improve; the other two KDE models had moderate performance (AUC < 0.8).

Discussion

Regionally trained models for invasive plants of management concern did not perform better than national models when evaluated with independent data from within the training region. Continental-scale models tended to outperform regional ones even when the number of regional training points was high (Fig. 3), supporting the general recommendation to use a broad spatial extent for training models of invasive species (Fitzpatrick et al. 2007; Broennimann and Guisan 2008). Mapped predictions from models trained on a focal region were more similar to continental scale predictions within that region, compared to extrapolated results from models trained in other regions (Fig. 2; Suppl. material 1: Fig. S4). When comparing area predicted as suitable by models trained on different geographic extents for the same target region, there was not a consistent pattern, but extrapolation led to more variable results (Fig. 3b). When interpolating, including training points beyond the focal region did affect predictions within that region, as we found differences in both the spatial pattern and the overall level of predicted suitability between continental and regional model outputs. The tendency for higher performance of continental models points to these modifications being generally positive for within-region model performance and indicates that models with a broader extent are less prone to swamp regional patterns than to usefully reduce model extrapolation.

For most species, we had insufficient data to estimate and evaluate a model in one or more of our focal sagebrush regions. For example, *V. dubia* lacked estimation data in the eastern sage region, and is established within only a small area of the Great Plains, where active EDRR efforts have yielded a large number of data points (Hart and Meador 2021). However, because our validation design utilized spatial strips to provide a more independent, and therefore more realistic, estimate of performance, we had insufficient regional validation points to assess model performance within the

Great Plains. In addition, strong spatial clustering of points early in an invasion, such as with *V. dubia* in the Great Plains, can reflect propagule pressure and the idiosyncrasies of dispersal, with many unoccupied locations due to dispersal limitation (Elith et al. 2010; Václavík and Meentemeyer 2012). Species distribution models trained on only a portion of a species' range are therefore likely to be less accurate in early invasion stages.

While this study focused on the geographic extent of estimation data, comparisons with previous work highlight how other modeling decisions shape predicted invasion risk. Here, we thresholded individual models in our ensembles based on a rule that categorized 10% of training presences as occurring in unsuitable habitat. This threshold rule is appropriate for EDRR activities where search is the end use of models and a targeted approach can focus search efforts towards areas with a relatively higher degree of suitability (Sofaer et al. 2019b). In contrast, Jarnevich et al. (2021) quantified invasion risk across management units, and therefore used a more precautionary approach, the 1st percentile threshold, to avoid minimizing invasion risk via errors of omission. In contrast to the 10th percentile threshold, the first percentile classifies 1% of training presences as being in unsuitable habitat and thus classifies a larger portion of a study area as suitable. Both thresholds are based only on presence information, as true absences are unavailable. The more targeted threshold used here resulted in a smaller extent of predicted suitability for the same species, and illustrates how different thresholds may be implemented depending on intended use (Sofaer et al. 2019b).

Our study varied the geographic extent of estimation data to compare continental and regional models. Our findings align with results for native species, where in the absence of *a priori* evidence for niche divergence, researchers recommended creating models across a species' range (Collart et al. 2021; Connor et al. 2019). However, we held predictor variables constant between geographies, and the inclusion of geospatial variables believed important for controlling a species distribution may produce a better model than one for a larger extent lacking that information. Indeed, our continental models do not include species' global ranges because we highly value predictor variables that are available for the U.S. but are not available, inconsistent, or of lower quality globally (e.g., information on soils). For these species we lacked information that would indicate we needed to vary predictors geographically.

Alternatives to regional models include allowing for non-stationarity in environmental responses via hierarchical modeling, geographically weighted regression (Osborne et al. 2007) or spatially-varying coefficient models (Gelfand et al. 2003). Hierarchical models can estimate both overall environmental responses and variation in those relationships among groups (e.g., via random slopes in a mixed modeling framework). Both regional and hierarchical modeling approaches typically require defining intraspecific groups, but little emphasis has been placed on the approaches used to define subpopulations, which should be well justified (Chardon et al. 2020). Here, we considered intraspecific divisions based on ecoregions; among native species, studies have diverged in whether splitting by ecoregion (Smith et al. 2019a) or by genetic similarity (Chardon et al. 2020) yields the best performance. Partial pooling, a hierarchical approach that incorporates multiple intraspecific groups within a single mixed

model, provides a method intermediate between splitting and lumping (Smith et al. 2019b). The research to define subpopulations takes time and resources which may not be available for many invasive species, particularly when time to action is critical in limiting invasion costs (Pergl et al. 2020). These alternatives add complexity and potentially require more resources to first define groupings appropriately and then create multiple or hierarchical models for a single taxon compared to a continental approach. There is a continuum of automation versus human time and insight in developing species distribution models (Young et al. 2020), from large extent models for suites of species using the same predictors for all models (e.g., Allen and Bradley 2016) to very detailed models for a single species (e.g., Smith et al. 2019b; Chardon et al. 2020). The best path forward depends on the objectives, data availability, a priori information about populations and species, and the available resources and timeline.

In selecting a modeling approach, it is important to distinguish between populations that have different limiting factors and populations that have different responses to environmental conditions. Across a species' range, it is typical that different limiting factors are suspected to constrain population growth; for example, an early macroecological hypothesis posited that biotic interactions more often defined southern range limits while abiotic conditions more often defined northern range limits (reviewed by Schemske et al. 2009). Cases where, for example, one area may be too dry while another is too cold can be handled by standard range-wide modeling approaches, as demonstrated by our study. It is only where the definition of 'too cold' varies among populations that more tailored or complex models may be warranted as highlighted by other studies of native species. Ideally, common garden experiments and similar tools would be used to test for differentiation but these types of studies for every invasive species would be time and cost prohibitive.

Conclusion

The degree of variation in responses to environmental conditions and the amount of data available underlie the selection of appropriate strategies for species distribution modeling (Fig. 4). Consistent responses to ecological conditions (e.g., Connor et al. 2019; Collart et al. 2021) or capturing a broader range of environmental conditions occupied by a species (e.g., Broennimann and Guisan 2008) support range-wide modeling (bottom right), while evidence for regional differentiation lends support to regional or hierarchical modeling methods where data are available (e.g., Chardon et al. 2020 upper right). However, there is a key tension between data availability and relevance for EDRR. Model outputs inform EDRR when they can be used to guide surveillance efforts and assess spatial patterns of invasion risk during a rapid response. Yet at these early stages of an invasion, there is necessarily little to no data on species' occurrences within the focal area or the data occur within such a small extent that model fitting is difficult (e.g., *V. dubia* in the Great Plains region; left side of Fig. 4). Regional models will typically be most relevant at later stages of an invasion, where

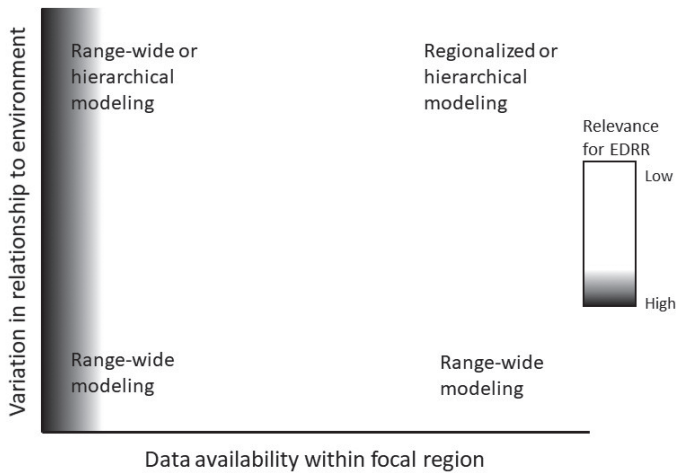


Figure 4. Conceptual depiction of the utility of different modeling methods and of the trade-offs between data availability within a focal region and relevance of model outputs for Early Detection and Rapid Response (EDRR) within that region. Range-wide modeling is appropriate where there is little variation in the relationship between a species' occurrence and environmental conditions. Where local populations are differentiated in their responses to the environment, hierarchical or regionalized models are expected to produce the most relevant predictions for within the region, and the selection among model types may depend on data availability, institutional capacity, and time horizon for delivering results. The relevance of model outputs for EDRR is high only very early in an invasion, when few data are available; therefore, range-wide modeling is expected to remain the primary tool used to anticipate habitat suitability for non-native species.

there has been more opportunity for population divergence, range filling, and data collection (moving from left to right within Fig. 4). Clear justification and communication of model assumptions between model producers, local knowledge holders, and decision-makers can help clarify what kinds of differences warrant regional or hierarchical models. Delayed actions may increase costs associated with invasions and decrease the ability to meet management goals for newly introduced species to a region (Ahmed et al. 2022). Regional models did not perform better than national models, and thus national models may have use to inform management decisions for early detection of invasive species.

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Supplementary material I

Tables S1–S4, Figures S1–S6

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Data type: Supplemental figures and tables.

Explanation note: **Table S1.** Total area (km²) and area of sagebrush habitat within each modeling region and its associated test strip. **Table S2.** Model assessment rubric from Sofaer et al. (2019b) for presence models for all species modeled. **Table S3.** Location summary including number of training points (and test strip points) in each spatial extent considered including the Great Basin, eastern sagebrush, the Great Plains where all three are limited to sagebrush, sagebrush in the western United States, and all locations in the continental United States. **Table S4.** The predictors considered in model development including the variable (name [unit of measure | spatial (cell) resolution | temporal resolution (if applicable)], description, and source from Engelstad et al. (2022)). **Figure S1.** For each species, map showing location point data colored by region (Great Basin sagebrush, eastern sagebrush, Great Plains sagebrush, other sagebrush, and outside of sagebrush). **Figure S2.** Proportion of locations for each species found in sagebrush habitat, as defined by the National Land Cover Dataset shrubland sagebrush rangeland fractional component product (Xian et al. 2015), within each region (continental United States, eastern sage, the Great Basin, and the Great Plains). **Figure S3.** Proportion of the total locations for each species recorded in each of six National Land Cover Dataset categories relative to the proportion of all invasive species locations in the same life-form (forb/ herb for first seven species; graminoid for last two species) to correct for sampling biases. Values above 1 indicate that more locations are found in that land-cover class relative to all other species. **Figure S4.** The 10-percentile ensemble map (values of 0 to 10) for each modeled training area (Great Basin, eastern sagebrush, Great Plains, all sagebrush, and Continental U.S. with inclusion determined by Table S3), with output restricted to the three regions (Great Basin, eastern sagebrush, Great Plains) for each of the nine species (a to i). Each map is overlaid by a transparent gray layer highlighting novel environments from the Multivariate Environmental Similarity Surface. **Figure S5.** Ranked variable importance for each model run (algorithm by background method) across species plotted for models trained on regions (all three combined), all sagebrush, and the continental U.S. Difference in AUC values from permutation of occurrence and background locations were

ranked across predictors by training extent, species, algorithm, and background method. **Figure S6.** Boyce index calculated for each region's test strip (columns) and the test strip 10 percentile ensemble model by species (x-axis) including the number of the species' occurrences within the test strip above the axis for the different models including a model trained using species' locations from sage (all sage), the continental U.S., the region matching the test strip region (matching region), or regions different from the test strip region (other region).

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Complex origins indicate a potential bridgehead introduction of an emerging amphibian invader (*Eleutherodactylus planirostris*) in China

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Abstract

Identifying the origins of established alien species is important to prevent new introductions in the future. The greenhouse frog (*Eleutherodactylus planirostris*), native to Cuba, the Bahamas, and the Cayman Islands, has been widely introduced to the Caribbean, North and Central America, Oceania and Asia. This invasive alien amphibian was recently reported in Shenzhen, China, but the potential introduction sources remain poorly understood. Based on phylogenetic analysis using mitochondrial 16S, COI and CYTB sequences, we detected a complex introduction origin of this species, which may be from Hong Kong, China, the Philippines, Panama and Florida, USA, all pointing to a bridgehead introduction. In

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addition, the nursery trade between the four countries or regions and mainland China from 2011 to 2020 was also significantly higher than other areas with less likelihood of introductions, which supported the molecular results. Our study provides the first genetic evidence of the potential sources of this emerging amphibian invader in mainland China, which may help develop alien species control strategies in the face of growing trade through globalization.

Keywords

Amphibian, biosecurity, bridgehead introduction, invasive species, phylogenetics

Introduction

Alien species invasion has been a major threat to global biodiversity, the economy and human health in the current era of Anthropocene (Pyšek et al. 2020). The rate of alien species invasion has substantially accelerated over the past century and is particularly concerning, because human-mediated introductions have reached unprecedented intensity (Seebens et al. 2017), which is projected to continue to increase in the next 50 years under sustained globalization (Seebens et al. 2021). Along the “introduction-establishment-spread” continuum of biological invasions (Blackburn et al. 2011), managing early risks at the introduction stage is considered one of the most effective mitigation strategies (Fournier et al. 2019). With the rapid development of molecular tools, identifying the origins of alien species based on molecular markers is critically important to understand the potential introduction process and thus prevent new potential invasions in the future (Estoup and Guillemaud 2010; Hudson et al. 2021), which has been widely used to develop control strategies for invasive alien species (Bai et al. 2012; Liebl et al. 2015; Wang et al. 2017).

Alien amphibians have been a particular conservation and environmental concern due to their substantial impacts on native species through predation, competition, and the spread of notorious wildlife diseases such as the chytridiomycosis panzootic (Liu et al. 2013; Kraus 2015). Additionally, there is general rapid anthropogenic introduction and dispersal of alien amphibians due to their close associations with human activities through the pet trade and aquaculture (Kraus 2009; Liu et al. 2014; Capinha et al. 2017; Capinha et al. 2020). The greenhouse frog (*Eleutherodactylus planirostris*), native to a few islands of the Caribbean, Cuba, and the Cayman Islands, the Bahamas, and the British Overseas Territory, is one such species that has been widely introduced in more than 10 states of the USA (Kraus et al. 1999; Somma 2022), Nicaragua (Heinicke et al. 2011), Mexico (Cedeño-Vázquez et al. 2014), Panama (Crawford et al. 2011), Jamaica (Pough et al. 1977), and Guam (Christy et al. 2007). In China, this species was first recorded in Hong Kong in 2000 (Lee et al. 2016), and then was found in Shenzhen in 2017 (Lin et al. 2017). This species is regarded as one of the most successful amphibian invaders with high population densities (e.g., 12,500 frogs/hectare in Hawaii (Olson and Beard 2012)) and diverse prey across a wide range of

invertebrates (e.g., 129,000 individuals/hectare/night (Olson and Beard 2012)), posing a high predation threat to insect populations and competition for food with other sympatric frogs in invaded areas (Kraus et al. 1999; Olson and Beard 2012). Notably, it is a direct-developing frog without a free-swimming larval stage and thus is highly adaptable to various terrestrial and aquatic habitats (AmphibiaWeb 2022). In addition, owing to its tiny body size (16–32 mm SVL), it is extremely well-suited to introduction to new ranges as a stowaway through imported plants and landscaping materials (Kraus and Campbell 2002). This unobserved and unintentional introduction makes it difficult to identify the source populations, which is nevertheless crucial for early prevention strategies to stop future introductions and may be possible with the aid of molecular tools (Ficetola et al. 2008).

Heinicke et al. (2011) first revealed the origin of invasive *E. planirostris* found in Florida might lie in western Cuba using a phylogenetic analysis method based on three gene markers (CYTB, RAG1 and PMOC). Later, studies of several invasive populations, such as those in Panama (2 samples, Andrew and Alonso 2011) and Hong Kong, China (2 samples, Lee et al. 2016), uncovered a bridgehead introduction that originated from the already established Florida populations, based on partial fragments of the 16S rRNA gene. Bridgehead introduction usually tends to cause loss of genetic diversity (Bertelsmeier and Keller 2018), which was evidenced by the observed extremely low genetic diversification of mitochondrial genes (CYTB, 16S rRNA gene or COI) from the populations in Mexico, the Philippines, Hawaii and Florida, USA (Cedeño-Vázquez et al. 2014; Contreras-Calvario et al. 2018; Que et al. 2020). These studies have provided striking examples of the possible introduction processes of different invasive populations of the greenhouse frog around the world. However, the potential origins of its emerging population in Shenzhen, China are still unclear. Shenzhen is located in the Guangdong-Hong Kong-Macao Greater Bay Area, which is an important region with a highly developed horticulture, gardening, and landscaping industry that provides an ideal opportunity for the introduction of greenhouse frogs through horticultural transportation. We speculated that Hong Kong, China, may be the most likely source of the Shenzhen population due to its close geographical location, similar climate, and frequent trade. However, the possibility of introduction from other countries and regions cannot be ruled out due to accelerating international trade, transportation, and infrastructure construction (Ding et al. 2008; Huang et al. 2012; Liu et al. 2019).

To fill this knowledge gap, we used phylogenetic analyses to explore the possible introduction routes of the Shenzhen population based on data from existing native and invasive populations and all available molecular sequences across the world. We aimed to provide timely insights into the source of this emerging frog invader in mainland China and contribute to the development of a sustainable Chinese biosecurity strategy against biological invasions, especially in regions such as the Guangdong-Hong Kong-Macao Greater Bay Area with growing social and economic activities for the prevention of biological invasions.

Methods

Field Sampling

Field surveys were conducted in Shenzhen, China on September 2–13, 2021. The third toe of the right hind foot from each postmetamorphosis greenhouse frog was clipped, and the tissue samples were preserved separately in 95% ethanol and stored at -20°C in the laboratory (Suppl. material 1: Table S1). A total of four samples were used for the phylogenetic analysis.

We obtained all published sequences of the greenhouse frog, including a total of 71 CYTB sequences, 13 COI sequences and 18 16S sequences from NCBI; All newly obtained sequences were deposited in GenBank (CYTB: OP554912-OP554915; COI: OP548504-OP548506, OP548508; 16S: OP547501, OP547876-OP547878) (Suppl. material 1: Table S1), along with one outgroup sequence from *Osteopilus septentrionalis* from Heinicke et al. (2011).

DNA extraction and amplification

Genomic DNA was extracted using the Universal Genomic DNA Kit (catalog no. CW2298M; Beijing, CoWinBiotech Co., Ltd, Beijing, China) following the manufacturer's instructions. To infer the potential geographic origin of the sampled individuals, we amplified one diagnostic mitochondrial marker corresponding to a portion of the protein encoding the 16S region, a portion of the protein encoding COI (Simon et al. 1994; Che et al. 2012), and a portion of the protein encoding the CYTB region, which were designed for this study using Primer 5 software (Lalitha 2000). The amplification conditions are shown in Suppl. material 2: Table S2. The PCR products were then separated by electrophoresis on 2% agarose gels. The resulting PCR products were directly sequenced by Beijing Liuhe Bgi Co., Ltd, Beijing, China, using the same primers for amplification. All sequences were tested for quality and calibrated manually using SeqMan in the LASERGENE 7.0 software package (Ahmed et al. 2016) to generate consistent sequences. All three genes obtained for each specimen sequence were compared with the available homologous sequences from GenBank (<https://www.ncbi.nlm.nih.gov/>) using the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with the default parameters. All the obtained consensus sequences were aligned using the default parameters in Clustal X 1.81 (Thompson et al. 1997), and then MEGA 7.0 was used to compare the sequences of each gene (Tamura et al. 2011).

Data analysis

Phylogenetic relationships of the greenhouse frog were reconstructed based on 16S sequence, COI sequence and CYTB sequence data using maximum likelihood (ML) and Bayesian (BI) analyses, respectively. The 'best' model of sequence evolution for the sequences was inferred using the Akaike Information Criterion (AIC) as implemented

in jModelTest 2 (Darriba et al. 2012). The best model was GTR+G based on 16S data. Since COI gene and CYTB gene were coding genes, based on this selection, we used DAMBE software (Xia and Lemey 2009) to screen each codon of COI and CYTB sequences, and used ModelTest software to calculate the optimal evolutionary model of each codon: TrNef for the first codon position of COI; F81 for the second codon position of COI; HKY+I for the third codon position of COI; TrH for the first codon position of CYTB; JC for the second and third codon position of CYTB. We ran an ML search using RAxML version 8.2.4 and assessed the results using nonparametric bootstrap resampling with 1000 replicates (Stamatakis 2014). Bayesian phylogenetic analyses were conducted using MrBayes 3.2 (Ronquist et al. 2012). According to the selected nucleotide replacement model, we implemented two independent runs at the default calorific value and used four MCMC chains each time, including three hot chains and one cold chain. All the data analyses were run for 1000 million generations and were sampled every 200 generations. Bayesian posterior probability values were estimated from the sampled trees that remained after the first 25% of trees were discarded as burn-in. Convergence was assured by an average standard deviation of split frequencies below 0.01 and accurate parameter estimates as indicated by estimated sample sizes above 200 and potential scale reduction factor values close to 1 in Tracer v1.5 (Rambaut and Drummond 2007). The haplotypes of each gene were calculated in DnaSP 5.10 (Librado and Rozas 2009) (Suppl. material 1: Table S1). Haplotype NETWORK graphs were generated using POPART 1.7 software (Ropiquet et al. 2015).

Validation of molecular analyses using nursery trade data

Considering the fact that the greenhouse frog was mainly transported through nursery trade (Kraus 2009; Lee et al. 2016; Lin et al. 2017), in order to validate the results based on molecular data, we further collected the available nursery trade volumes imported from all the candidate countries or regions to mainland China from different databases such as the United Nations Commodity Trade Statistics database (<https://comtrade.un.org/>, Suppl. material 3: Table S3). Since the greenhouse frog was first discovered in Shenzhen, China, in 2017 (Lin et al. 2017), we collected a total of 10-year bilateral trade data from 2011 to 2020. We then used a Kruskal-Wallis test to compare the 10-year trade volume between mainland China and the countries or regions with the most likely sources including Hong Kong, China, the Philippines, Panama, and Florida, USA and the other areas with less introduction likelihood based on molecular data.

Results

Our 16S sequence data set consisted of 550 bp from all 4 individuals in a matrix of 137 variable sites. Combined with the results of the haplotype network, ML and BI trees constructed from the sequences of three genes indicated that the Shenzhen population may be from Hong Kong, China, the Philippines, Panama and Florida, USA.

For instance, the samples from Shenzhen were found to share the same haplotype with populations in Hong Kong, China, the Philippines, Panama, and Florida, USA, based on the sequences of the 16S gene (Fig. 1). Similarly, the COI sequence data set consisted of 596 bp from all 4 individuals in a matrix of 149 mutation locus, which also supported the conclusion that the greenhouse frog in Shenzhen may originate from the populations in the Philippines and Panama, which share the same haplotype (Fig. 2). The CYTB sequence data set consisted of 635 bp from all 4 individuals in a matrix of 174 mutation locus. However, the CYTB sequences showed that the greenhouse frog from Shenzhen had a distinct haplotype, and it was closest to the haplotype shared by Florida, Hawaii, USA, Matanzas in Cuba, and the Philippines, which suggested that the individuals of Shenzhen population may have originated from one or more of these areas (Fig. 3). The bilateral trade analysis further showed that the volumes of importing nursery trade in 2011–2020 from the molecular-based likely source areas including Hong Kong, China, the Philippines, Panama, and Florida, USA, was indeed significantly higher than the other areas with less likelihood of introductions (Bahamas; Cuba; Cayman Islands, UK; Turks and Caicos Islands, UK; Jamaica; Nicaragua; Hawaii, USA) (Kruskal-Wallis test, $P = 0.0001175$).

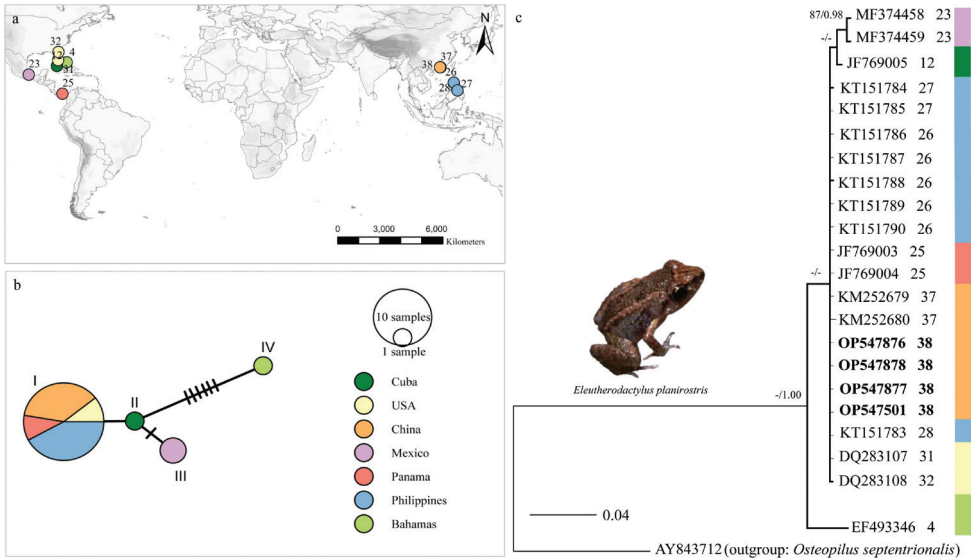


Figure 1. **a** sample distribution and results for the mitochondrial 16S sequences; each number represents a different sequence location **b** relationship among the network of haplotypes of the greenhouse frog (*Eleutherodactylus planirostris*). The size of the circles is proportional to haplotype frequency. Each color represents a locality/country **c** bayesian/maximum Likelihood phylogenetic tree of the greenhouse frog inferred from a fragment of the 16S gene. “-” denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap support (BS < 70%). Colors indicate mitochondrial lineages, and each color represents a different country: Cuba (dark green), Bahamas (aqua), USA (light yellow), Mexico (purple), Panama (red), the Philippines (blue), and China (orange). *E. planirostris* image: from <http://www.amphibiachina.org/>.

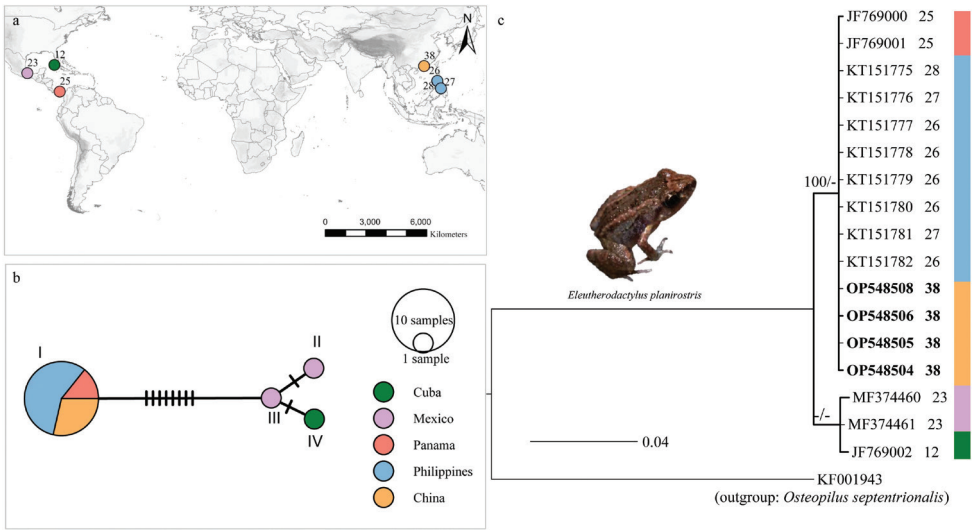


Figure 2. **a** sample distribution and results for the mitochondrial COI sequences; each number represents a different sequence location **b** relationship among the network of haplotypes of the greenhouse frog (*Eleutherodactylus planirostris*). The size of the circles is proportional to haplotype frequency. Each color represents a locality/country **c** bayesian/maximum likelihood phylogenetic tree of the greenhouse frog inferred from a fragment of the COI gene. “-” denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap support (BS < 70%). Colors indicate mitochondrial lineages, and each color represents a different country: Cuba (dark green), Mexico (purple), Panama (red), the Philippines (blue), and China (orange). *E. planirostris* image: from <http://www.amphibiachina.org/>.

Discussion

To the best of our knowledge, this is the first quantitative study on the potential introduction source of the emerging global amphibian invader (the greenhouse frog, *E. planirostris*) in mainland China. According to the standard of Heinicke et al. (2011), our results using 16S and COI identified two lineages of the greenhouse frog (i.e., an eastern lineage from eastern/central Cuba, the Bahamas and the Cayman Islands, and a western lineage from western Cuba and Florida, USA), and the samples we obtained from the Shenzhen population belong to the western lineage (Figs 1–3). In the western lineage, previous studies suggested that the greenhouse frog in Hong Kong, China and the Philippines originated in Florida (Lee et al. 2016; Que et al. 2020); our result thus indicates that the original source of the greenhouse frog in Shenzhen may be Florida, USA, which warrants further investigation using more samples collected at each site and based on more powerful genetic information, such as genomes generated by next-generation sequencing techniques (Blumenfeld et al. 2021). Mitochondrial DNA used in our present study may, in particular, have a limited ability to track recent invasions because this marker requires accumulation of variation over long timescales (Browett et al. 2020). The whole-genome or molecular marker with high mutation rate (such as microsatellites

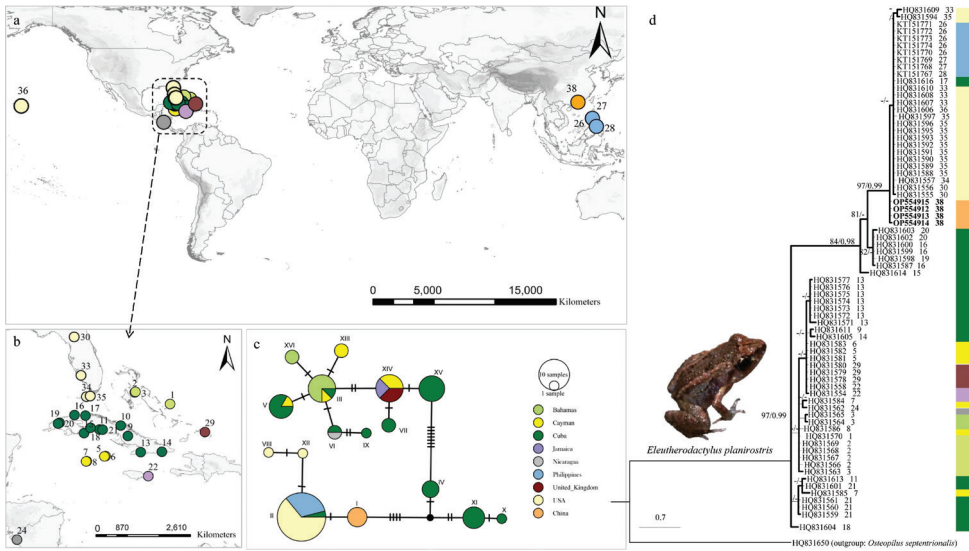


Figure 3. **a, b** sample distribution and results for the mitochondrial CYTB sequences. Each number represents a different sequence location **c** relationship among the network of haplotypes of the greenhouse frog (*Eleutherodactylus planirostris*). The size of the circles is proportional to haplotype frequency. Each color represents a locality/country **d** bayesian/maximum likelihood phylogenetic tree of the greenhouse frog inferred from a fragment of the CYTB gene. “-” denotes low support by Bayesian posterior probabilities (BPP < 95%) and bootstrap support (BS < 70%). Colors indicate mitochondrial lineages, and each color represents a different country: Cuba (dark green), Bahamas (aqua), Cayman (yellow), USA (light yellow), Jamaica (dark purple), UK (dark red), Nicaragua (gray), the Philippines (blue), and China (orange). *E. planirostris* image: from <http://www.amphibiachina.org/>.

and single nucleotide polymorphisms, SNPs) can help improve the analysis accuracy (Ellegren 2004; Li 2011; Rius et al. 2015). However, data on these higher resolution markers of the greenhouse frog are not available yet from Genbank. We suggest that further investigations with closer international collaborations can obtain cross-border sample collections to facilitate a more robust identification of introduction sources of this global frog invader. We therefore encourage future studies using microsatellites or SNP markers to support or refute our conclusions. Nevertheless, our analyses based on the volume of the bilateral nursery trade between different candidate countries or regions and mainland China supported our molecular analysis as the trade volumes of several higher likely source areas such as Florida, USA, Philippines, Hong Kong, China and Panama, is indeed higher than other areas with lower introduction likelihood.

Interestingly, all the candidate source populations identified in the present study are located in the nonnative ranges of the greenhouse frog, supporting the potential bridgehead introductions, which have been observed in several other invasive populations around the world (e.g., Andrew and Alonso 2011; Lee et al. 2016). The bridgehead effect can often reduce the genetic diversity of subsequent invasive populations after experiencing bottleneck events (Blumenfeld et al. 2021). However, bridgehead populations

have been found to effectively reduce inbreeding depression by removing those deleterious alleles (Facon et al. 2011) and increasing the rate of adaptive evolution of those traits, promoting their invasion of novel ranges (Bertelsmeier and Keller 2018). Therefore, the bridgehead effect of alien species has been observed to be increasingly common across taxa, especially for invasive insects (Correa et al. 2019; Javal et al. 2019; Sherpa et al. 2019). The increasing bridgehead introduction modes of alien species imply the importance of applying transport network theory by incorporating trade and animal translocations to obtain a full picture of the alien species introduction process (Banks et al. 2015).

In general, the global invasive populations of the greenhouse frog have always been reported in large cities or in localities with great commercial nursery trade volumes (Contreras-Calvario et al. 2018), which is one important pathway involved in the human-mediated movement of alien herpetofauna (Kraus 2009). There has been a long history of nursery trade in the Pearl River Delta region of Guangdong Province and Hong Kong, China. For example, in 2015, over 100,000 kg of plants or parts of plants were exported from Hong Kong, China, to tropical or subtropical countries/ or regions, including Australia, mainland China, Macau, China, Malaysia, Singapore, Thailand, Taiwan, China and Vietnam (Census and Statistics Department, Hong Kong SAR 2015). Such a high volume of horticulture might, therefore, have posed a high risk of alien species invasions, especially the alien herpetofauna. We therefore suggest that more strict quarantine policies and early warning frameworks should be made to prevent the continued incursions of alien species.

Conclusion

We provided genetic evidence on the potential introduction sources of an emerging amphibian invader (*E. planirostris*) in China, which is further validated using international nursery trade of different alternative countries or regions with mainland China. We observed multiple introduction candidate sources, which all indicated a bridgehead introduction. Overall, these findings demonstrate the complexity of the greenhouse frog introductions from their already invaded ranges to China and stress the importance of developing stricter monitoring strategies to mitigate the stowaway introduction of this global amphibian invader from different areas worldwide.

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Supplementary material I

Table S1

Authors: Yanhua Hong Yanhong He Zhiqiang Lin, Yuanbao Du, Shengnan Chen, Lixia Han, Qing Zhang, Shimin Gu, Weishan Tu, Shengwei Hu, Zhiyong Yuan, Xuan Liu
Data type: occurrences, phylogenetic

Explanation note: Sequence location and distribution for genetic samples of *Eleutherodactylus planirostris* used in this study. We obtained all published sequences of the frog, including a total of 71 CYTB sequences, 13 COI sequences and 18 16S sequences from NCBI.

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Link: <https://doi.org/10.3897/neobiota.77.83205.suppl1>

Supplementary material 2

Table S2

Authors: Yanhua Hong Yanhong He Zhiqiang Lin, Yuanbao Du, Shengnan Chen, Lixia Han, Qing Zhang, Shimin Gu, Weishan Tu, Shengwei Hu, Zhiyong Yuan, Xuan Liu

Data type: phylogenetic

Explanation note: Primers and PCR conditions used in this study. The amplification of 16S, COI and CYTB conditions.

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Supplementary material 3

Table S3

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Data type: word

Explanation note: The available nursery trade volumes from 2011 to 2020 were imported from all the candidate regions to mainland China from different databases.

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Invasive *Drosophila suzukii* outnumbers native controphics and causes substantial damage to fruits of forest plants

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Abstract

Impacts of biological invasions are diverse and can have far-reaching consequences for ecosystems. The spotted wing drosophila, *Drosophila suzukii*, is a major invasive pest of fruits, which negatively affects fruit and wine production. However, little is known about the ecological impact of this fly species on more natural ecosystems it has invaded, such as forests. In this study, we investigated the use of potential host plants by *D. suzukii* at 64 sites in different forest communities in Switzerland from mid-June to mid-October 2020. We examined more than 12,000 fruits for egg deposits of *D. suzukii* to assess its direct impact on the plants. We recorded symptoms of fruit decay after egg deposition to determine if *D. suzukii* attacks trigger fruit decay. In addition, we monitored the drosophilid fauna with cup traps baited with apple cider vinegar, as we expected that *D. suzukii* would outnumber and potentially outcompete native controphics, especially other drosophilids. Egg deposits of *D. suzukii* were found on the fruits of 31 of the 39 potential host plant species studied, with 18 species showing an attack rate > 50%. Overall, fruits of *Cotoneaster divaricatus* (96%), *Atropa bella-donna* (91%), *Rubus fruticosus corylifolius* aggr. (91%), *Frangula alnus* (85%) and *Sambucus nigra* (83%) were attacked particularly frequently, resulting also in high predicted attack probabilities that varied among forest communities. Later and longer fruiting, black fruit colour, larger fruit size and higher pulp pH all positively affected attack rates. More than 50% of the plant species showed severe symptoms of decay after egg deposition, with higher pulp sugar content leading to more severe symptoms. The high fruit attack rate observed was reflected in a high abundance and dominance of *D. suzukii* in trap catches, independent of forest community and elevation. *Drosophila suzukii* was by far the most abundant species, accounting for 86% (81,395 individuals) of all drosophilids. The abundance of *D. suzukii* was negatively associated with the abundance of the native drosophilids. Our results indicate that the invasive *D. suzukii* competes strongly with other frugivorous species and

that its presence might have far-reaching ecosystem-level consequences. The rapid decay of fruits attacked by *D. suzukii* leads to a loss of resources and may disrupt seed-dispersal mutualisms through the reduced consumption of fruits by dispersers such as birds.

Keywords

Alien species, drosophilid, ecological impact, frugivore, fruit decay, host plant range, insect

Introduction

Biotic exchange and subsequent invasions by non-native species in natural and human-modified ecosystems are among the greatest threats to biodiversity worldwide (Wilcove et al. 1998; Pyšek et al. 2020). They can have far-reaching consequences for ecosystems (Vilà et al. 2010), including their functioning (Mack et al. 2000; Ehrenfeld 2010), and for human well-being (Shackleton et al. 2019), and they can cause extraordinary economic costs (Pimentel et al. 2005; Diagne et al. 2021). The perception and recognition of impacts of biological invasions, as well as how they are measured, are diverse and depend on the variables and scales considered (Jeschke et al. 2014). While assessments of economic impacts are measured in terms of economic costs, ecological impacts are evaluated as measurable changes to the properties of an ecosystem by a non-native species. However, the ecological impact of an invader is not easy to define or quantify, due to the context dependency of impacts, the variation in the per capita effect within and across species, and the complex interactions between invaders and their biotic and abiotic environments (Pyšek and Richardson 2010; Ricciardi et al. 2013).

Invasive non-native species may affect native species on the level of individuals (e.g. fecundity, mortality), populations (e.g. abundance, genetic diversity), communities (e.g. species richness and composition, trophic structure) and ecosystems (e.g. nutrient cycling, physical habitat, overall structure and function) (Parker et al. 1999). The impact of a non-native species on native populations and communities varies greatly in temporal (Strayer et al. 2006) and spatial (Mollot et al. 2017) terms and depends critically on the abundance and trophic position of the invasive species (Bradley et al. 2019). This explains why the extent of ecological impact varies greatly between invaders (Kumschick et al. 2015; Lapin et al. 2021).

Insects make up a large proportion of introduced species (DAISIE 2009; Seebens et al. 2017). They are usually introduced accidentally, rarely reach large populations, and/or are often not noticed. However, a small minority become highly abundant and ecologically significant (Liebhold and Tobin 2008). Impacts of non-native insects on native species and ecosystems have many potential mechanisms, but only a very small proportion of non-native insects have been studied regarding their ecological impacts (Kenis et al. 2009). While direct impacts through mechanisms such as predation or competition are more obvious, indirect impacts, such as apparent and exploitative competition or alteration of interactions between native species, often remain unexplored (Traveset and Richardson 2006; White et al. 2006). For example, a meta-analysis of the effects of the invasions of the Argentine ant (*Linepithema humile*) showed that areas with *L. humile*

had 92% fewer native ant seed dispersers than areas where *L. humile* was not present. In addition, the meta-analysis indicated that *L. humile* did not replace native seed dispersers in their functional role, as rates of seed removal and seedling establishment were lower in the presence of *L. humile* (Rodríguez-Cabal et al. 2009). A disruption of seed-dispersal mutualisms affects seedling recruitment, species populations and distributions, plant-community composition, and gene flow (Howe and Smallwood 1982). Such indirect effects of non-native species on plant species can have far-reaching ecological consequences.

For frugivorous insects, the most significant effect on plants is thought to be indirect, namely the reduction of seed dispersal far from the mother plant by vertebrates (Sallabanks and Courtney 1992). Among frugivorous insects, *Drosophila suzukii* (Matsumura 1931; Diptera: Drosophilidae), also known as the spotted wing drosophila, is of particular importance. It is an invasive *Drosophila* species originating from South-east Asia (Asplen et al. 2015). The presence of the species outside of its native range was first recorded in Japan (Matsumura 1931), 1980 in Hawaii (Leblanc et al. 2009) and in 2008 synchronously in southwestern USA (Hauser 2011) and southern Europe (Cini et al. 2012). The species has spread rapidly and is now widespread on the Asian, European, and North and South American continents (dos Santos et al. 2017). The family Drosophilidae comprises more than 3,900 species, with more than 1,500 of the described species belonging to the genus *Drosophila*. These small flies are widespread in a variety of climates and environments throughout the world (Markow and O'Grady 2005a; Bächli 2021). To date, around 36 species of the genus *Drosophila* and more than 30 species of other genera of the family Drosophilidae are distributed in Switzerland (Bächli et al. 2004; personal communication G. Bächli 2021). The habitat of most of these species is mainly restricted to forest areas (so-called wild species). In contrast, a few species of *Drosophila*, so-called domestic species, mainly occur in settlements and are mostly cosmopolitan in distribution (Atkinson and Shorrocks 1977; Shorrocks 1977; Burla and Bächli 1991). Oviposition and larval development of *Drosophila* are usually restricted to decaying organic resources such as slime flux, fruits, flowers and mushrooms (Markow and O'Grady 2008), but the degree of substrate specialisation varies widely, from generalists to obligate specialists (Mitsui and Kimura 2000b; Markow and O'Grady 2005b; Anholt et al. 2020). This variability is also related to host location, host acceptance, and host use (Markow 2019), with chemical recognition playing a crucial role (Anholt 2020). Most *Drosophila* show some degree of fidelity in oviposition site selection, which often depends more on the decay state of the substrate, than on the identity of the plant or fungus (e.g. Kambysellis and Heed 1971; Nunney 1990; Karageorgi et al. 2017). For example, *D. suzukii* prefers fruits that are more intact (Kienzle and Rohlf 2021), firmer (Sato et al. 2021) and in an earlier maturation stage (Dweck et al. 2021) compared with *D. melanogaster*, another frugivore. *Drosophila* species inoculate their oviposition substrate with microorganisms. They are considered important vectors, especially of yeasts but also of bacteria, and transport viable microbes to new substrates where they grow (Gilbert 1980; Rohlf and Hoffmeister 2005; Stamps et al. 2012; Hamby and Becher 2016). Adults and larvae feed mainly on the microbes that decompose organic material, but also on the decomposing material itself (Markow and O'Grady 2008). Flies additionally benefit

from the dispersal of such microbes, as they obtain signals from their metabolic products for finding sugar resources (Madden et al. 2018).

Drosophila suzukii exploits resources that usually consist of small separate units and are patchy and ephemeral, i.e. they support only one generation. The temporal and spatial constraints of these unpredictable resources may favour generalists (Jaenike 1990). Niche breadth usually correlates positively with geographical range size (Slatyer et al. 2013), but does not necessarily explain invasion success (Carscadden et al. 2020). Following this general rule, the temporal and spatial niche breadth of the invasive *D. suzukii* have been shown to be relatively large compared with other *Drosophila* species in Japan (Yamamoto 1984). Besides using fruits as its preferred substrate for oviposition, it has also been found to develop on fungi (Kimura 1976) and can even complete development on chicken manure (Stockton et al. 2019). Within its invasive range, *D. suzukii* can use the fleshy fruits of many crops and wild plants (e.g. Poyet et al. 2015; Arnó et al. 2016; Kenis et al. 2016). In the year 2020, 198 plant species representing 40 families were already confirmed as host plants (Little et al. 2020), so the fly can be considered extremely polyphagous (sensu Normark and Johnson 2011).

Polyphagous herbivores (or generalists) are more impacted by plant toxicity than specialists (Ali and Agrawal 2012), as specialisation in phytophagous insects is thought to assist in the evolution of adaptations to overcome plant defences (Ehrlich and Raven 1964). However, *D. suzukii* hardly needs to specialise to overcome plant defences. On the one hand, because fleshy fruits are generally intended to be eaten by vertebrates (Lei et al. 2021), the pulp of such ripe fruits is rather easily digested (Cipollini 2000). On the other hand, the association with microbes may help with detoxification and digestion (Douglas 2009, 2015). Fruits share particular volatiles across plant species (Prasanna et al. 2007). As a resource specialist utilising particular plant structures, i.e. fruits, with predictable odour cues, it may not be crucial for *D. suzukii* to specialise on particular plant species in order to increase host location, because olfactory responses to substrate-relevant volatiles of a resource instead of a plant species enables coping with a much narrower range of odours (e.g. Becher et al. 2012; Cunningham and Zalucki 2014; Cunningham et al. 2016).

Drosophila suzukii's attacks on the fruits of agricultural crops cause considerable economic damage to fruit growers through yield losses and the need to take measures to prevent attacks and minimise damage (Bolda et al. 2010; Knapp et al. 2020). Research on *D. suzukii* as a fruit crop pest is therefore ongoing and diverse. In recent studies, the occurrence of *D. suzukii* outside agricultural crops has been investigated to assess pest pressure from adjacent habitats. Woodlands have been shown to be suitable refugia and overwintering habitats (Briem et al. 2016; Pelton et al. 2016; Thistlewood et al. 2018; Wallingford et al. 2018) and can provide a large reservoir of hosts that produce fruits, ensuring continuity of resource availability over time (Poyet et al. 2015; Arnó et al. 2016; Elsensohn and Loeb 2018; Thistlewood et al. 2019). Therefore, large numbers of *D. suzukii* can be expected in forests, as has also been shown in trapping case studies (e.g. Briem et al. 2018; Haro-Barchin et al. 2018; Santoiemma et al. 2018), and population densities are likely to be more constant and higher than in semi-open habitats such as agricultural landscapes. So far, almost no research has been conducted

to address the ecological impact of this fly on the ecosystems it has invaded. Roche et al. (2021) highlighted how *D. suzukii* may produce ecological changes to eastern forests in the USA. Invasion by *D. suzukii* may have consequences for food resources and, consequently, on species with which they compete for fruits. Competitive interactions, i.e. competition for shared resources or interference between species, generally increase with increasing phylogenetic relatedness (Li et al. 2015) and functional similarity (Dick et al. 2017) between the invader and the native species. Accordingly, *D. suzukii* could affect native drosophilids due to two main mechanisms. First, *D. suzukii* can use a wide range of substrates for oviposition, which gives it a competitive advantage over native drosophilids (Karageorgi et al. 2017; Silva-Soares et al. 2017). Unlike other drosophilids that use damaged and overripe fruits, *D. suzukii* has an enlarged, serrated ovipositor which makes it possible for females to pierce the skin of fruits (Atallah et al. 2014). It thus can use undamaged fruit, i.e. it can occupy fruits earlier than other drosophilids. Second, according to the enemy release hypothesis, a lower parasitisation rate leads to a larger population size (Keane 2002; Shea 2002), and parasitisation is an important cause of mortality for drosophilid larvae (Janssen et al. 1988; Fleury et al. 2009).

In this study, we assessed the use of potential host plants in forests by examining egg depositions of *D. suzukii*, reflecting its direct effect on the plants. Since there is a diverse abundance and varying availability of host plants within a plant community, which affects the choice of host plants for *D. suzukii*, we selected different forest communities and forest edges as study sites. We addressed the following questions: (1) Are there differences in the potential host plants of *D. suzukii* growing in different forest communities? (2) To what extent are the potential host plants attacked by *D. suzukii*, and what are the factors influencing the attack rates?

Due to the large numbers of *D. suzukii* trapped in previous studies, we expected that this species would outnumber and potentially outcompete native controphics, especially drosophilids. To test this hypothesis, we trapped drosophilids during the study period and addressed the following questions: (3) What proportion of drosophilid individuals are *D. suzukii*? (4) Does the proportion of *D. suzukii* differ among forest communities? (5) Does the abundance of *D. suzukii* affect the abundance of other drosophilids?

Materials and methods

Study area

We conducted our study in the adjacent cantons of Zug (47.092440–47.218600°N, 8.407940–8.680231°E; elevation 400–1200 m a.s.l.) and Zurich (47.163290–47.365790°N, 8.424865–8.687711°E; 440–1165 m a.s.l.) in Switzerland from June to November in 2020 (Fig. 1). In both regions, the annual average temperature ranges between 4–6 °C at higher elevation sites to 10–12 °C at lower elevation sites and the annual precipitation ranges between 1100–1300 mm and 1700–2000 mm at sites in the canton Zug and 900–1100 mm and 1100–1300 mm at sites in the canton Zurich (climate norm values 1991–2020; Federal Office of Meteorology and Climatology

MeteoSwiss; extracted from <https://map.geo.admin.ch/>). We selected the forest communities 7a (*GALIO ODORATI-FAGETUM TYPICUM*), 19a (*ABIETI-FAGETUM LUZULETOSUM*) and 26f/g (*ACERI-FRAXINETUM MERCURIALIDETOSUM*) (see Ellenberg and Klötzli 1972) for our study, as they include a comparable range of fruiting plants. In addition, we investigated forest edges, as they serve as important habitat for a large range of fruiting plants. We examined the three forest communities at four sites ≥ 1 km apart, from 400 to 1165 m a.s.l. At each site, we selected four 25 m \times 25 m plots of the particular forest community from the available grid cells (also 25 m \times 25 m) in a stratified random design, using a vegetation mapping GIS in the canton of Zurich (Kanton Zürich 2020) and vegetation maps in the canton of Zug. We applied the following criteria in selecting plots: (1) distance between plots ≥ 150 m, (2) distance to the forest edge ≥ 150 m (due to the shape of the area, only a distance of ≥ 70 m was possible at the site “Zollischlag”). We measured the distances between sites, between plots and to the edge of the forest using the mapping platform of the Swiss Confederation (www.map.geo.admin.ch) and subsequently checked them in the field. We reviewed the forest community classification in the field based on indicator plant species. We defined the centre of the plots as the tree nearest to the actual centre point of the 25 m \times 25 m area. We moved a selected plot to the adjacent grid cell if the area was temporarily unstocked ($n=2$), was covered with logging residues ($n=1$), or had no characteristics of the respective forest communities ($n=3$). We investigated forest edges at four sites with four plots each in the canton of Zug. We selected two sites at low elevations (400–600 m a.s.l.) and two at high elevations (800–1000 m a.s.l.). We defined these plots as areas of 12.5 m \times 50 m along the forest edge (instead of 25 m \times 25 m). Where possible, the forest edges were orientated in different cardinal directions. Forest edge sites were ≥ 1 km apart and plots were separated by ≥ 250 m. All sites were located in managed forests.

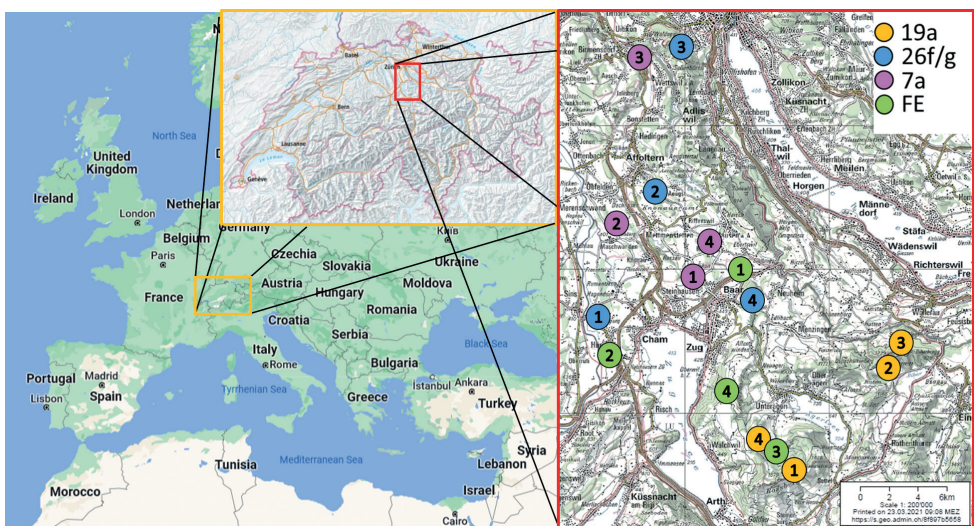


Figure 1. Map showing the locations of the 16 sites (forest communities 7a, 26f/g and 19a; forest edges (FE)) (European map data 2021 Google maps, Switzerland map data 2021 Swiss Confederation).

Field surveys of potential host plants

We considered any fleshy-fruited species, characterised by a high fruit water content, a potential host plant. We used the term “fruit” for all fruit types (i.e. berries, drupes, aggregate drupelets, aggregate nutlets and pomes), independent of the tissue of origin of the pulp. We also examined the aril of European yew (*Taxus baccata*) for egg deposition and included it under the term “fruit” below.

We carried out the field surveys in all plots during six observation sessions (interval of 20 days) over the study period, starting on 22/06/2020 and ending on 15/10/2020: 4 plant communities (3 forest communities + forest edge) \times 4 study sites \times 4 plots (64 plots) \times 6 periods = 384 assessments.

We mapped all potential reproducing host plant species in each plot (25 m \times 25 m or 12.5 m \times 50 m = 625 m²) and noted its occurrence. We used an estimated dominance value to determine the area (m²) covered by a plant species, and used this value and the recorded height to calculate the plant species' volume. For each potential host plant at each field survey per plot, we estimated the seasonal phenology and the number of ripe fruits present, assigning maturation stage between 0 and 2, corresponding to the majority of plant individuals: 0 = no ripe fruits, 0.25–0.75 = before main fruit maturity (some ripe fruits), 1.0–1.25 mainly ripe fruits, 1.5–1.75 = after main fruit maturity (more over-ripe, fermented fruits than ripe fruits), 2 = no more fruits. Since fruit ripening is usually associated with a change in colour, we used colour change as an indicator of the maturity of the examined fruits. In some species, such as European fly honeysuckle (*Lonicera xylosteum*), rowan (*Sorbus aucuparia*) or alder buckthorn (*Frangula alnus*), changes in fruit flesh firmness during ripening were not advanced at the time of colour change. We judged these fruits to be ripe when they also softened. After maturity, fruits enter senescence (the period during which chemical synthesis pathways give way to degradation processes). As an indicator of the “overripe” stage, we used loss of moisture, which becomes visible as a loss of turgor. At the end of the field survey, we calculated the maturity period with the unit of half a month and estimated the amount of fruit, both per potential host plant species per plot. The exact method used for these estimates depended on the species and was based on counts (e.g. all observed fruits, fruits per square metre, fruits per individual plant, or infructescence). We then extrapolated counts to the plot level. The number of fruits was likely underestimated by this approach and was thus a rather conservative estimate. Fruits that were removed, destroyed or lost due to drought stress near the end of the ripening process were not subtracted from the estimated numbers (see Suppl. material 1: Table S1 for month of fruit maturity, number of fruits, and occurrence (volume) of potential host plant species of *Drosophila suzukii* in the study plots).

Near the plots and in the same forest communities, we additionally examined native potential host plants that occurred in less than three plots (*Lonicera alpigena*, *Taxus baccata*, *Prunus spinosa*, *Crataegus laevigata*, *Lonicera periclymenum*, *Viburnum lantana*). We conducted this additional investigation to estimate the average attack rate of plants with infrequent occurrence on the plots, but we excluded these fruits from the statistical analyses.

European brambles (*Rubus* L. subg. *Rubus*) are taxonomically divided into three sections: *Rubus*, *Corylifolii* and *Caesii* (with one species, *Rubus caesius*). The most

abundant species in our plots were *R. hirtus* agg. (sect. *Rubus*), occurring in the plots of forest community 19a, and *R. villarsianus* (sect. *Corylifolii*), occurring in the plots of forest communities 7a and 26f/g. Especially at the forest edges, but also in the forest communities 26f/g and 7a, more than one species of the sections *Rubus* and/or *Corylifolii* occurred in the plots. We did not identify the described and named species of the sections *Rubus* and *Corylifolii* in the plots and therefore used the term *Rubus fruticosus corylifolius* agg. as the taxonomic unit.

Investigation of fruit attacks by *Drosophila suzukii*

We examined ripe fruits at an accessible height (up to 2 m) for egg deposition using a hand lens (10× magnification). If we observed at least one *D. suzukii* egg on the fruit, we considered the fruit “attacked”, regardless of the number of egg deposits or larvae (for literature on the number of eggs per host plant, see e.g. Lee et al. 2015; Poyet et al. 2015; Olazcuaga et al. 2019; for development potential in different hosts see e.g. Arnó et al. 2016; Kenis et al. 2016; Tonina et al. 2016). We examined at least 10 fruits per population or individual of a potential host plant per plot, and we removed the examined fruits from the plants. If multiple individuals of a plant species occurred in the plot, we examined several individuals. In the case of large populations of brambles (*R. fruticosus corylifolius* agg.) or bilberry (*Vaccinium myrtillus*), i.e. covering > 25% of the plot area, we randomly selected 5 areas of 1 m² for investigating egg deposition. Egg deposition by *D. suzukii* is detectable for only a limited time, due to decay of the fruit substance, rotting, development of the larvae, feeding by other animals, or secondary infections such as grey mould. No larvae in a damaged fruit does not allow inference of non-infestation. Further, damaged fruits may in turn be used as egg-laying substrate by other drosophilids. Therefore, we used only ripe, undamaged fruits to study egg deposition on potential host plants.

We investigated the fruits of European holly (*Ilex aquifolium*) for egg deposition after the field surveys because they had not yet reached fruit maturity during the study period. To check the oviposition activity of *D. suzukii*, we simultaneously examined fruits of the European dwarf elder (*Sambucus ebulus*) and brambles (*Rubus fruticosus corylifolius* agg.) occurring near the European hollies under investigation. We did not examine European mistletoe (*Viscum album*) and common ivy (*Hedera helix*), which were also present in the plots, for *D. suzukii* attacks, as their fruit ripening period fell well outside our study period. The ripe fruits of the wild strawberry (*Fragaria vesca*) often showed numerous feeding marks and damage, such that egg deposition by *D. suzukii* could rarely be detected. We therefore decided not to investigate attacks further and excluded wild strawberry from the analyses. We could confirm its use as a host, however, as adults hatched from collected fruits and we found numerous drosophilid larvae in otherwise intact fruits.

Fruit and decay traits

We compiled fruit traits of the investigated plant species from Herrera (1987), Snow and Snow (1988), Eriksson and Ehrlén (1991), and Stiebel (2003) and from databases

(eFloras 2021, TRY 2021). When the records of fruit traits were comparable or convertible, we included the data from several literature references and used the average value. We included the fruit traits: type, colour, diameter (mm), mass (g), water content of the pulp (%), sugar content of the pulp (glucose and fructose, % of dry mass), lipid content of the pulp (% of dry mass), protein content of the pulp (% of dry mass), pH, persistence of individual fruits (days) and “attacked by non-dispersal frugivores” (%). In addition, we used the amount of ripe fruit and the maturation start and duration, based on our field assessments (see section “Field surveys of potential host plants”), as fruit traits in the analyses.

Fruit decay is a complex natural phenomenon that is co-determined by numerous conditions. It occurs as a result of physical and chemical damage, enzymatic digestion, and especially microbial activity. The fruit skin, which serves as an external barrier, is damaged by the egg deposition of *D. suzukii*. We documented the fruit response to egg deposition, i.e. decay, to determine if attacks trigger fruit decay. We recorded the following symptoms of fruit decay: oviposition scar, denting, oozing of pulp, reduction of fruit substance/loss of shape, and colour change. We categorised the symptoms as mild (1), moderate (2) or severe (3).

Adult trapping

At each site of the forest communities (including forest edges), we installed two baited (80–100 ml) transparent plastic cup traps covered by a red lid with 3 mm diameter entry holes (Profatec AG, Malans, Switzerland) to trap Drosophilidae including *D. suzukii*. As an attractant, we used a mixture of apple cider vinegar and water (3:1; apple cider vinegar IP-Suisse, Denner AG, 8045 Zurich, Switzerland; acetic acid 50 g/l) with 1–2 drops of liquid soap (Oecoplan Abwaschmittel, Coop, 4002 Basel, Switzerland) per 5 dl. We positioned the traps at a height of 1.2–1.5 m on branches of plants that do not bear fleshy fruits, mostly beech (*Fagus sylvatica*), at a distance of 150–200 m from the field survey plots. We installed the traps from 22/06/2020 to 07/07/2020, during the first session of the egg deposition assessment. At the forest edges, we placed the traps approx. 3 m inside the forest (from the shrub belt). We kept the traps in the same positions throughout the experiment and visited them on the same dates as the egg deposition assessments. Therefore, each sampling period of adult drosophilids also lasted 20 days.

During each sampling session, we removed the contents of the traps and preserved them in ethanol, and we renewed the bait. We sorted the trap content into different taxa, which we identified to different taxonomic levels. We identified the non-native *D. suzukii* and other drosophilids to the species level using the identification key of Bächli et al. (2004). “Domestic species”, in particular *Drosophila simulans* and *Drosophila immigrans*, and other non-native species (e.g. *Drosophila curvispina* and *Chymomyza amoena*) were occasionally trapped. Other non-native drosophilid species accounted for < 1% of all trapped individuals. Therefore, we used the term “native drosophilids” for all drosophilids other than *D. suzukii*. We identified other Diptera to the family level following Oosterbroek (2006). See Suppl. material 2: Table S2 for a list of the

trap catches. Since trap captures of a single trap during our study period should not be considered as replicates, especially because of varying phenology in different species (e.g. Bahder et al. 2016), we used the totals of trap captures per trap for the analysis.

Statistical analyses

We carried out all statistical analyses using R version 4.0.2 (R Core Team 2020). We tested all models for multicollinearity using the ‘check_collinearity’ function of the *performance* package (Lüdtke et al. 2021). We performed a test for dispersion, zero inflation and residual diagnostics with the *DHARMA* package (Hartig 2022).

Differences in fruiting plant communities

To test whether the composition of the plant communities differed between the forest communities with respect to the plants with fruits relevant for *D. suzukii*, we conducted non-metric multidimensional scaling (NMDS) based on a Bray-Curtis distance matrix (function ‘metaMDS’, package *vegan*; Oksanen et al. 2020). We defined the abundance of plant species as the estimated number of fruits per study plot and season, square-root transformed. We used the ‘adonis’ function in *vegan* (permutational multivariate analysis of variance, PERMANOVA) to test for statistical differences between the forest communities.

Attack rate and probability of fruits of different plant species

We applied a binomial generalised mixed effects model (package *glmmTMB*; (Brooks et al. 2017)) to test for differences in fruit attacks between forest communities (including forest edges) and plant species. The ratio of attacked to unattacked fruits per study plot and time of recording (cbind(N attacked, N not attacked)) was the response variable, whereas the forest community, the plant species, the elevation (m a.s.l.), the number of ripe fruits, the fruit maturity status, and the canton were the predictor variables. We included study plot as a random term to account for the nested study design. We used an additional observation-level random factor due to dispersion issues.

We simplified our model stepwise by excluding the factor with the highest *p*-value and comparing the two models with the *anova* function based on a χ^2 -test. If the more complex model did not differ significantly in model performance from the simpler model, we used the latter. This procedure resulted in the exclusion of canton and elevation (height_NN) from the final model.

We calculated the attack probabilities (LS-means \pm SE) of the different plant species in the forest communities and plotted them using the *emmeans* package (Lenth 2022) based on the final model.

The role of fruit traits in fruit attack and decay

We explored whether fruit traits could explain observed differences in attack rates. We first used NMDS to illustrate the fruit trait space of the 39 studied plant species. See

the section “fruit traits” for a list of the fruit traits considered. We treated each plant species in each forest community separately to relate it to attack rate and phenology, which both differed between forest communities for a given plant species. We used the Gower dissimilarity coefficient (Gower 1971) with Podani’s (Podani 1999) extension for ordinal variables to create a distance matrix from our fruit trait data (‘gowdis’ function in the *FD* package (Laliberté and Legendre 2010; Laliberté et al. 2014)).

We performed NMDS (with two axes) on the Gower distance matrix using the ‘metaMDS’ function in the *vegan* package (Oksanen et al. 2020). For illustration, we plotted attack rates of the fruits with different circle sizes and the phenology (month of maturation start) in different colours. We plotted traits post-hoc using the ‘envfit’ function in *vegan*, with 1000 permutations.

We used a binomial generalised mixed effects model (package *glmmTMB*; (Brooks et al. 2017) to test whether fruit attacks were related to fruit traits. The ratio of attacked to unattacked fruits per study plot and time of recording (cbind(N attacked, N not attacked)) was the response variable, whereas fruit availability (amount of fruit per plot), start of ripe fruit availability and duration of ripe fruit availability (both 0.5 month resolution), fruit colour, fruit diameter, sugar content of the pulp, and pulp pH were the predictor variables. We excluded fruit type and mass as well as water content of the pulp because of multicollinearity issues, and lipid and protein content of the pulp, as well as persistence and “attacked by non-dispersal frugivores”, because they had too many missing values. We defined study plot and plant species as random terms to respect the nested study design and the repeated measures on plant species. We used an additional observation-level random factor due to dispersion issues. We standardised all quantitative variables to zero mean and unit variance using the ‘decostand’ function in the *vegan* package (Oksanen et al. 2020) to allow comparisons of effect sizes.

We used a general linear model (‘glm’) with a Poisson distribution to test whether fruit traits determined the decay status of the fruits after the attack by *D. suzukii*. We used the sum of decay traits (see section “fruit and decay traits”) as the response variable and fruit traits (fruit diameter, pulp pH, pulp water, sugar, lipid and protein content) as predictors. We excluded fruit type and mass because of multicollinearity issues. We standardised all quantitative variables to zero mean and unit variance using the ‘decostand’ function in the *vegan* package (Oksanen et al. 2020) to allow comparisons of effect sizes.

Effects of *D. suzukii* on native Drosophilidae

To test for the effects of forest community and canton on the abundance of *D. suzukii* adults captured in traps, we used a binomial generalised mixed effects model (package *glmmTMB*; (Brooks et al. 2017) with the ratio of *D. suzukii* and native Drosophilidae (cbind(N *D. suzukii*, N native Drosophilidae)) as response variable and study plot as random term. We calculated the predicted proportions of *D. suzukii* (LS-means \pm 95% CI) of the Drosophilidae species caught in the traps in the forest communities and plotted them using the *emmeans* package (Lenth 2022) based on the above model. To predict the abundance of native Drosophilidae as a function of the abundance of *D. suzukii*, we used a generalised mixed effects model with a Poisson distribution (package *glmmTMB*;

Brooks et al. 2017) and defined forest community and canton as additional fixed effect and study plot as random term. We then plotted the predicted effect of *N. D. suzukii* on *N* native Drosophilidae using the *effects* package (Fox and Weisberg 2018).

For all GLMs and GLMMS, we performed a type II Wald chi-square test using the R package *car* (Fox and Weisberg 2019). For most analyses we plotted the standardised estimates (effect sizes) using the ‘plot_model’ function in the sjPlot package (Lüdtke et al. 2021).

Results

Attacks on potential host plants

The composition of the potential host plants of *D. suzukii* differed among forest communities (PERMANOVA, $F=5.432$, $R^2=0.22$, $P=0.001$). The effect of forest community in structuring the plant communities is illustrated by the clustering of the forest communities in the ordination plot, except for the plant community of the study site of a former alluvial forest (site 26_1), which was more similar to forest community 7a than to 26f/g (Fig. 2). The greatest differences in the potential host plant composition were evident between forest communities 19a and 26f/g (pairwise adonis, $R^2=0.21$, $P=0.001$). Forest community 26f/g and the forest edge community were most similar ($R^2=0.07$, $P=0.08$).

At the study sites of forest community 19a, 7 potential host plant species with an estimated 34,000 fruits were recorded. At the study sites of the forest community 26f/g, there were 30 potential host plant species and an estimated 60,200 fruits; in forest community 7a there were 21 potential host plant species and 140,500 fruits, and at the forest edge study sites 32 potential host plant species and 161,000 fruits were observed (Suppl. material 1: Table S1).

Of the 39 potential host plants investigated, attacks were observed on 31 species. Overall, fruits of *Cotoneaster divaricatus* (96%), *Atropa bella-donna* (91%), *Rubus fruticosus corylifolius* aggr. (91%), *Frangula alnus* (85%) and *Sambucus nigra* (83%) were attacked particularly frequently (Suppl. material 1: Fig. S1, proportions of fruits attacked by *Drosophila suzukii* per plant species, separated by forest community). Based on the standardised assessments within the plots, the attack rates differed significantly between plant species and forest communities (Table 1), with forest edges exhibiting particularly high rates, followed by 7a. *Lonicera alpigena*, *Taxus baccata* and *Atropa bella-donna* had the highest probability of being attacked (Fig. 3). Larger numbers of ripe fruits and fruits in a later maturation stage corresponded to higher attack rates (Table 1). Canton and elevation did not have a significant effect on the attack rate and were dropped during model simplification.

The fruit trait space covered by the studied fruits was quite large (Fig. 4, left). The ordination plot illustrates that the attack rate differed greatly among plant species and was determined by the maturation start. Later maturation and longer availability of ripe fruits had a positive effect on attack rate (Table 2, Fig. 4 right). In addition, fruit

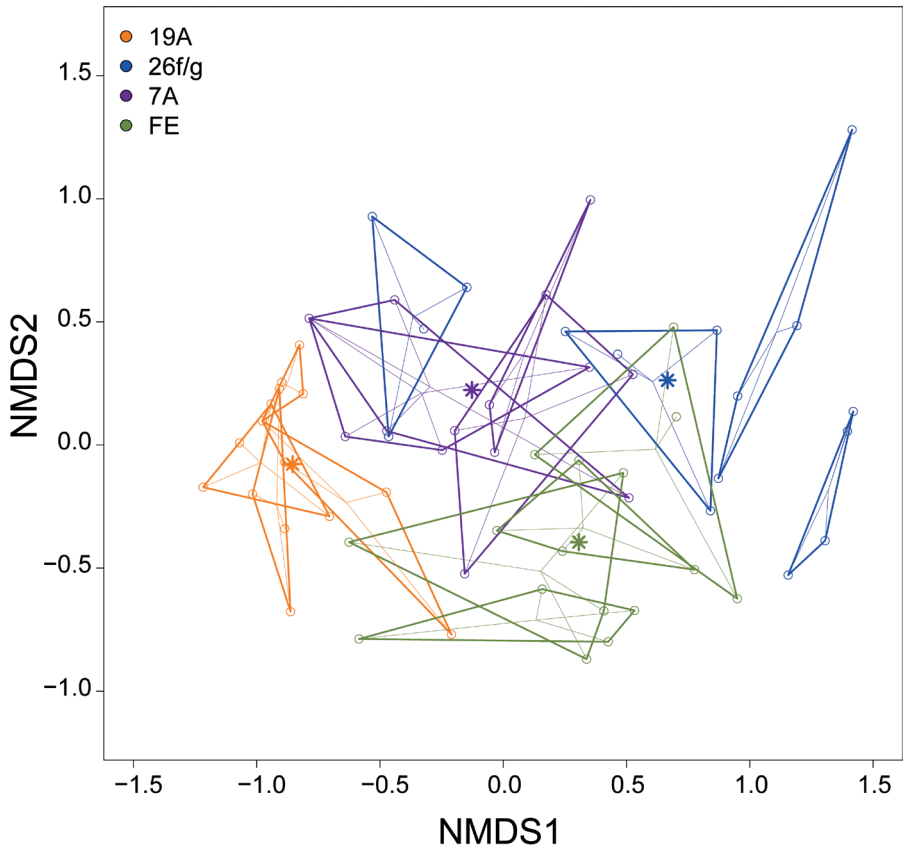


Figure 2. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances, showing the host plant composition with its estimated number of fruits for *Drosophila suzukii* at the study sites (four plots each) of the different forest communities. Different forest communities are indicated by different colours. Each dot represents the community in one plot. All the plots in a given site are connected by thick lines, and centroids are indicated by thin lines. The asterisks indicate the centroids of the forest communities. Stress=0.145 (k=3). Forest communities: 7a – *GALIO ODORATI-FAGETUM TYPICUM*, 19a – *ABIETI-FAGETUM LUZULETOSUM*, 26f/g – *ACERI-FRAXINETUM MERCURIALIDETOSUM* (see Ellenberg and Klötzli 1972), FE = forest edge.

Table 1. Results of the binomial generalised mixed effects model (glmmTMB) testing the drivers of attack rates of fruits by *Drosophila suzukii*. Plot nested in study site was defined as a random term. Results of the Wald Chi-square test are given. For continuous variables the direction of the effect (+) is given.

	<i>Chi</i> ²	Degrees of freedom	<i>P</i>
Forest community	34.385	3	<0.001
Plant species	396.861	36	<0.001
Number of ripe fruits	7.513	1	0.006125 (+)
Status of maturation	69.353	1	<0.001 (+)
R ² conditional	0.840		
R ² marginal	0.826		

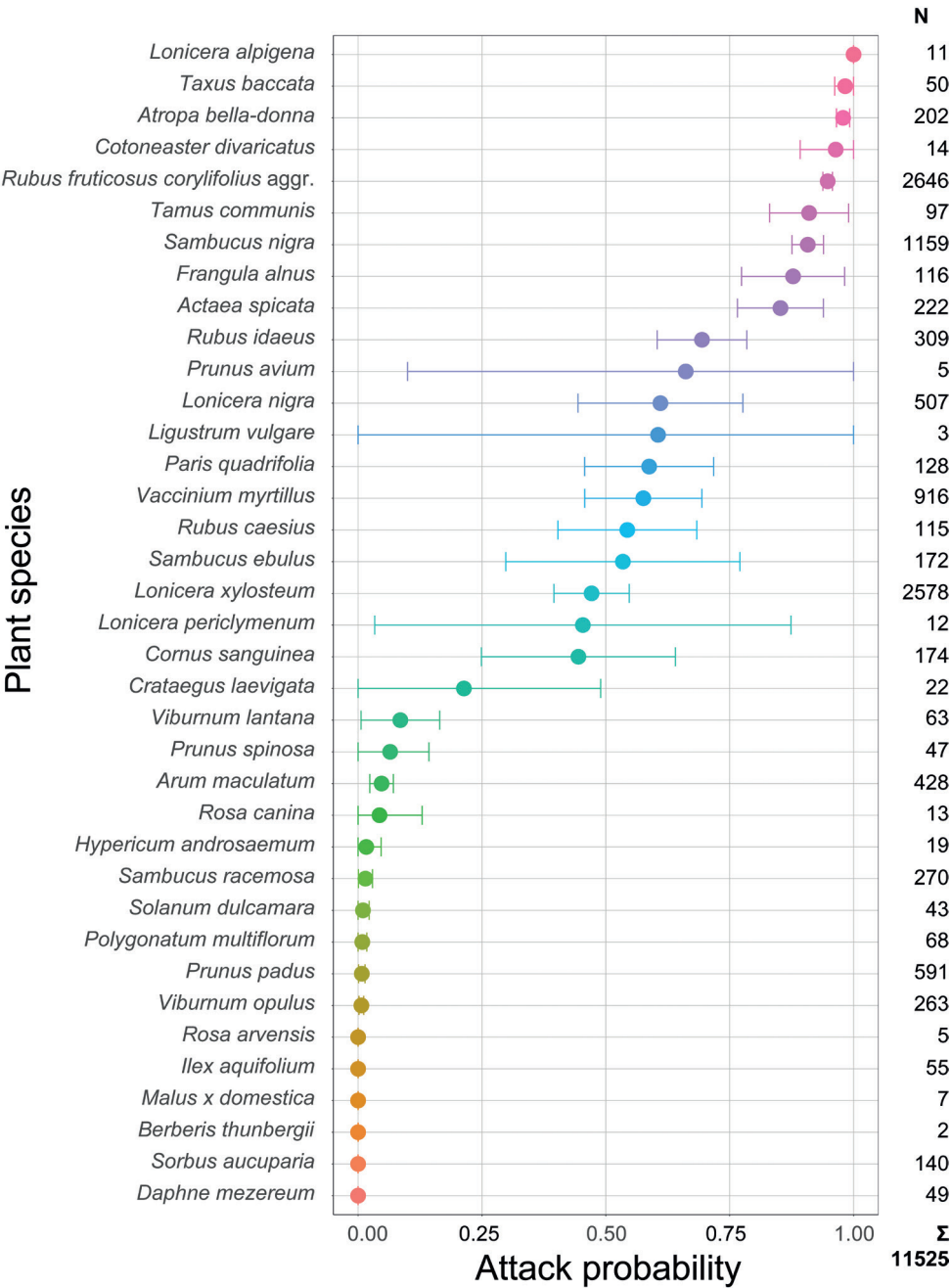


Figure 3. Attack probabilities of fruits of different plant species by *Drosophila suzukii*. Model-derived (for model results, see Table 1) probability estimates are shown (LS-means \pm SE, back-transformed from the logit scale to the original probability scale). Only fruits observed within the plots were considered. N refers to the number of observations. For average attack rates per species, including fruits outside the plots, see Suppl. material 1: Fig. S1.

colour affected attack rate, with orange fruits having a lower attack rate than black fruits, and blue and red ones tending to be attacked less than black fruits. In addition, larger fruits and fruits with a higher pulp pH had higher attack rates.

Of the studied fruit traits, only pulp sugar content affected fruit decay status after an attack by *D. suzukii*, with a higher sugar content leading to more severe symptoms of decay (Table 3, Fig. 5).

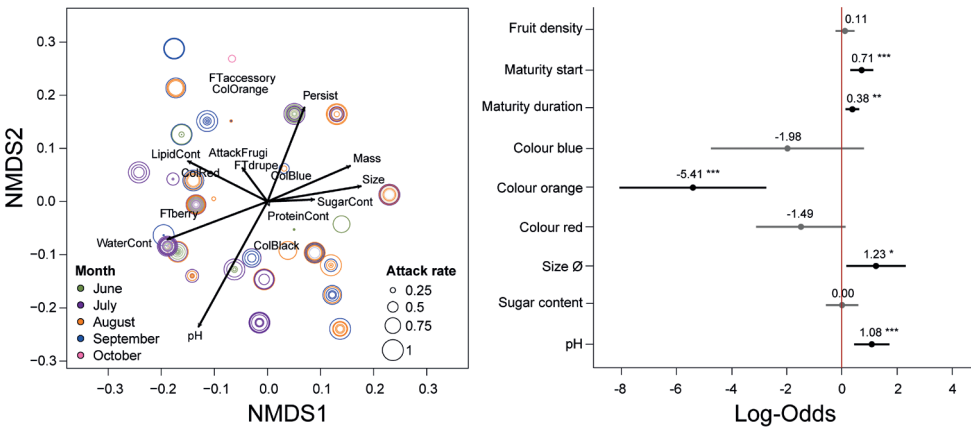


Figure 4. Left: Ordination plot of a non-metric multidimensional scaling (NMDS) analysis illustrating the fruit trait space of the 39 studied plant species (stress-value 0.198). The centre of each circle represents the position of a plant species in the fruit trait space. Attack rates by *Drosophila suzukii* are represented by the size of the circles, and the maturation start month of a plant species in a forest community is illustrated by the colour. FT = fruit type, Col = fruit color, Cont = content of the pulp, Persist = persistence of individual fruits (days), AttackFrugi = “attacked by non-dispersal frugivores” (%). Right: Effect size plot of a generalised linear mixed effects model (binomial glmmTMB) testing the effects of fruit traits, including fruit density and maturation start and duration (both 0.5-month resolution), on the attack rate by *D. suzukii*. Please note that some traits shown in the figure were excluded from the model due to multicollinearity issues or a large number of missing values (see Materials and Methods). The asterisks indicate significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2. Results of a binomial generalised mixed effects model (binomial glmmTMB) testing the effects of fruit traits on the attack rates of fruits by *Drosophila suzukii*. Plot nested in study site, as well as plant species and an observation-level random factor, were included as random terms. Results of a Wald Chi-squared test are given. For continuous variables the direction of the effect (+) is given.

	<i>Chi</i> ²	Degrees of freedom	<i>P</i>
Amount of ripe fruit	0.375	1	0.5404119
Maturation start	11.334	1	<0.001 (+)
Maturation duration	8.967	1	0.0027495 (+)
Fruit colour	16.944	3	<0.001
Fruit size Ø	5.024	1	0.0249951 (+)
Pulp sugar content	<0.001	1	0.9955224
Pulp pH	11.090	1	<0.001 (+)
R ² conditional	0.678		
R ² marginal	0.461		

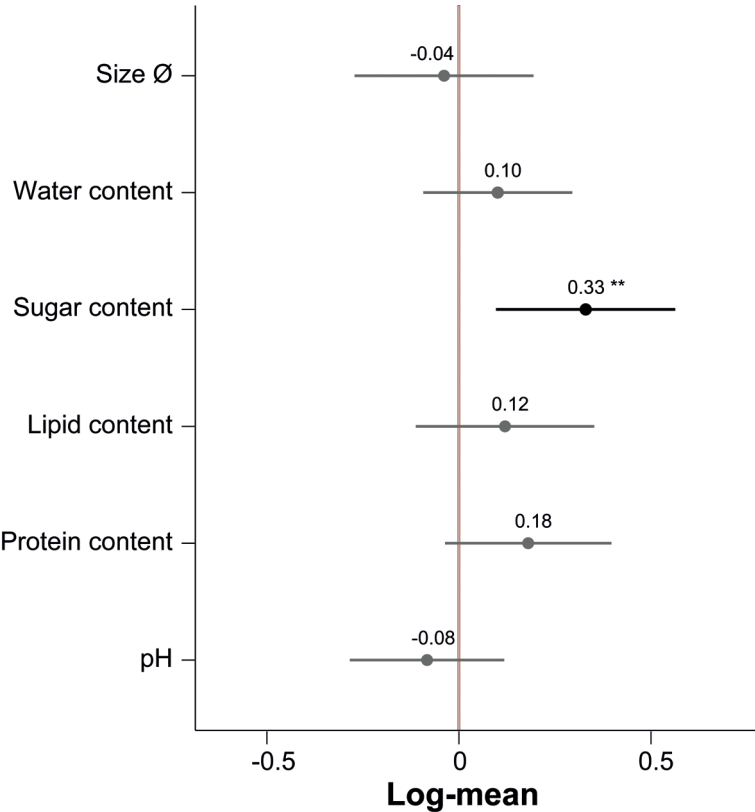


Figure 5. Effect size plot of a generalised linear model (poisson glm) testing the effects of fruit traits (size of the fruits and contents and pH of the pulp) on the decay status of fruits attacked by *Drosophila suzukii*. The asterisks indicate significance level: **p<0.01.

Table 3. Results of a generalised linear model (poisson glm) testing for the effects of fruit traits on fruit decay status after an attack by *Drosophila suzukii*. Results of a Wald Chi-squared test are given.

	<i>Chi</i> ²	Degrees of freedom	<i>P</i>
Fruit size Ø	0.108	1	0.742903
Pulp water content	1.0408	1	0.307632
Pulp sugar content	7.967	1	0.004763 (+)
Pulp lipid content	1.004	1	0.316309
Pulp protein content	2.591	1	0.107493
Pulp pH	0.655	1	0.418313
R ² Nagelkerke	0.547		

Adult trapping

During the investigation period, the traps captured 99,366 insects from four orders, each with at least 75 individuals (97,965 Diptera, 751 Hymenoptera, 552 Coleoptera, 75 Dermoptera) (160 traps). Hemiptera, Lepidoptera, Blattodea and Thysanoptera

occurred less frequently. Within the Diptera, species from 27 families were found, with Drosophilidae accounting for almost all observed individuals (97%; 94,624 individuals), followed by Anisopodidae (1%; 1399), Heleomyzidae (<1%; 486), Mycetophilidae (<1%; 310), Phoridae (<1%; 278), Scatopsidae (<1%; 247), Sciaridae (<1%; 127), Chloropidae (<1%; 118), and Dryomyzidae (<1%; 103) etc. *Drosophila suzukii* was by far the most abundant species (82% of all trapped insects, 81,395 individuals) and accounted for 86% of all drosophilids (Suppl. material 2: Table S2).

The species composition of drosophilids in the different forest communities differed, e.g. *D. alpina* was only found in forest community 19a and species of the genus *Amiota* were mainly caught in forest community 26f/g. However, the drosophilid assemblages were dominated by *D. suzukii*, independent of forest community (Fig. 6). We trapped 21,758 (84%) *D. suzukii* and 4,117 (16%) native drosophilids in the forest community 19a, 17,031 (81%) and 4,054 (19%) in 26f/g, 15,708 (86%) and 2,528 (14%) in 7a, and 26,871 (91%) and 2,530 (9%) at the forest edge. The ratio of *D. suzukii* to native drosophilids did not differ significantly between forest communities (Wald Chi-squared test, $\chi^2=3.053$, DF=1, $P=0.384$) and cantons ($\chi^2=0.036$, DF=1, $P=0.849$). The proportion of *D. suzukii* predicted by the models (purple symbols) was between 0.81 and 0.95 (Fig. 6).

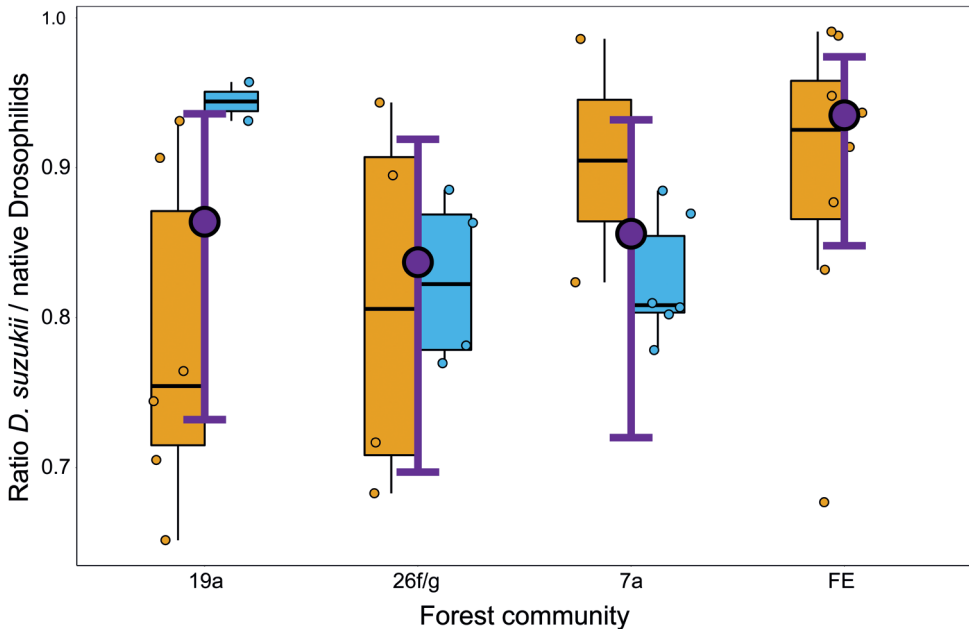


Figure 6. Ratio of *Drosophila suzukii* to native Drosophilidae species adults captured in traps in different forest communities. The boxplots (median, 25%/75% quantiles, min and max values) show raw values for the cantons Zug (orange) and Zurich (blue). Model-derived predicted estimates are shown in purple (LS-means \pm 95% CI, back-transformed from the logit scale to the original probability scale). Forest communities: 7a – *GALIO ODORATI-FAGETUM TYPICUM*, 19a – *ABIETI-FAGETUM LUZULETOSUM*, 26f/g – *ACERI-FRAXINETUM MERCURIALIDETOSUM*) (see Ellenberg and Klötzli 1972), FE = forest edge.

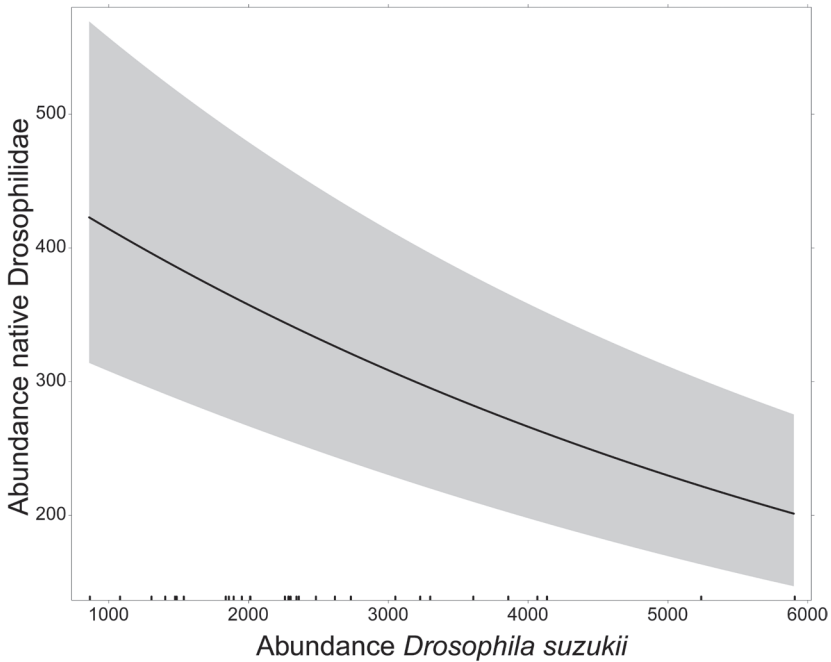


Figure 7. Predicted abundance of other drosophilids as function of the abundance of *Drosophila sukukii* (LS-means \pm 95% CI) from a generalised linear model including forest community and canton as covariates.

The abundance of *D. sukukii* significantly affected the abundance of native drosophilids captured in the traps (Wald Chi-squared Test, $\text{Chi}^2=74.072$, $\text{DF}=1$, $P<0.001$). The higher the abundance of *D. sukukii*, the smaller the abundance of native drosophilids predicted (Fig. 7). The forest community ($\text{Chi}^2=2.445$, $\text{DF}=3$, $P=0.485$) and the canton ($\text{Chi}^2=0.192$, $\text{DF}=1$, $P=0.661$) did not affect the abundance of native drosophilids.

Discussion

Drosophila sukukii, an invasive parasite of forest fruits, attacked 31 species from 15 different plant families (79% of all potential host plants investigated) in the forests of cantons Zug and Zurich. This not only confirms the broad host plant spectrum described in previous studies, but points towards a broad host use for reproduction. A broad host plant spectrum was to be expected, as *D. sukukii* is specialised on fruits and not on plant species per se. Furthermore, *D. sukukii* has been shown to have high plasticity in its host choice (Kienzle et al. 2020; Little et al. 2020). Our results suggest that a large number of fruiting plant species in forests are affected by *D. sukukii*, with likely far-reaching consequences for ecosystem processes (e.g. plant recruitment, resource availability for frugivores) and services (e.g. berry picking).

What influences attacks on the forest plants studied?

The observed broad host plant spectrum raises the question of what limits the host spectrum. Species of *Rosa* or *Sorbus*, which bear rather hard fruits, were hardly infested. We assume that the pulp and skin firmness act as a barrier to egg deposition, as insects have a limited ability to penetrate the skin of the fruit and to lay an egg in hard fruit flesh. It has been reported that the probability of oviposition increases as the force required to penetrate fruit skin decreases (Burrack et al. 2013; Lee et al. 2016). Further, within grape cultivars and single berries, berry skin resistance was found to explain the oviposition preferences of *D. suzukii* (Entling et al. 2019; Tonina et al. 2020). The fact that *Ilex aquifolium* was not infested was probably because its maturity coincides with the reproductive diapause of the fly (Grassi et al. 2018). We found that the red or blue, soft-skinned fruits of *Polygonatum* (Asparagaceae) were hardly used as a host, although this genus is widespread in the fly's area of origin (eFloras 2021). *Drosophila suzukii* may not detect all of the numerous potential host plants because its ability to process multiple sensory inputs is limited (Bernays 2001). Polyphagous herbivores have been shown to be attentive to the volatiles that are shared across host species (Silva and Clarke 2020). As the Asparagaceae are relatively distantly related to other host plants, the fruits and their microbial association with Asparagaceae species might not share certain volatiles with other host plants, making them undetectable to the fly.

The fruits of our 39 potential forest host plants differed in several characteristics that affect host location and oviposition site selection (Markow 2019), reflected by differences in observed attack rates in our study. Within the host plant range of *D. suzukii*, preferences (disproportionate use of potential host plants, i.e. egg deposits) have also been shown for crops in agricultural systems (Lee et al. 2011; Burrack et al. 2013; Olazuaga et al. 2019) and for wild and ornamental non-crop hosts (Poyet et al. 2015) when fruits were exposed to flies in laboratory assays. These preferences may be due to numerous characteristics of the fruit, such as colour, diameter, shape, volatile compound content, firmness, skin texture, or chemical composition, such as sugar content. In the field, several plant characteristics and the diverse abundance and fluctuating availability of host plants, as well as other biotic and abiotic factors of the host site, may also affect the choice of hosts, which makes comparisons of our findings with laboratory assays difficult.

Our non-metric multidimensional scaling (NMDS) indicated high variability in fruit traits and phenology in relation to attack rate. The attack rate varied among fruit colours, with black fruits being most frequently attacked. *Drosophila suzukii* has previously been shown to use colour as visual cue, but that colour contrast rather than colour appearance may be of greater importance (Little et al. 2019). While visual cues are of some importance, especially in behaviours such as courtship (Anholt et al. 2020), the olfactory and gustatory systems of *Drosophila* are crucial for host localisation and selection (Anholt 2020). We therefore expect that other factors correlated with colour might be decisive. For example, the fruit type “pome” or “hips”, which usually has high pulp firmness, mostly had a low attack rate and was often orange or red in colour. Larger fruit size also had a positive effect on attack rate. This could be because a larger

surface area, especially with aggregated fruits such as brambles, makes it easier for *D. suzukii* to find a preferred substrate, e.g. one without damage (mechanical or due to infection) or in an earlier maturation state. We also found that the attack rate increased with higher pulp pH (from pH 2.89 to 6.48). During host location and oviposition site selection, the fly can hardly detect a pH value, and thus this effect might be indirect. Microbes can act as the interface between insect herbivores and their hosts (Jansson et al. 2008; Hansen and Moran 2014; Ljunggren et al. 2019). *Drosophila suzukii*, like other *Drosophila*, is strongly attracted to volatiles produced by microorganisms in particular yeasts associated with fruits (Wright 2015; Hamby and Becher 2016; Bueno et al. 2020). It has also been shown that yeast volatiles, not fruit volatiles, mediate attraction and oviposition in *D. melanogaster* (Becher et al. 2012). In general, within the range of the fruit pulp pH, yeast and bacteria thrive better at higher pH values (Barth et al. 2009; Howell 2016). Fruits with a higher pH may host more microbes and could therefore be more attractive and easier to locate. In addition, moulds (filamentous fungi), an important competitor of *Drosophila* larvae (Wertheim et al. 2002; Rohlf et al. 2005; Trienens et al. 2010), generally exhibit better growth in a lower pH environment (Zhao et al. 2020). This could have led to a lower attractiveness for *D. suzukii* in our study, as decreased egg deposition in response to grey mould (*Botrytis cinerea*) was observed in a recent study (Chakraborty et al. 2022).

Host preferences are considered evidence of specialisation (Loxdale and Harvey 2016). In this case, however, we would expect phylogenetic relatedness in the preferred hosts, which we did not find, as the plant species with the highest observed attack rate belong to very distinct plant clades. Since the larvae feed in particular on microbes, host preferences, unlike those of folivorous insects, are determined more by the quality of the substrate in promoting the yeasts and bacteria associated with *D. suzukii* than by the phylogeny of the host plants. Our results suggest that *D. suzukii* responds to common cues from multiple host species, as well as specific cues from individual host species, as has been shown for other polyphagous herbivores (Silva and Clarke 2020). Other polyphagous herbivores also exhibit preference hierarchies for their hosts (e.g. Clarke et al. 2011; Wang et al. 2017), which may change through learning (West and Paul Cunningham 2002). Preferences should evolve toward maximising offspring fitness (Jaenike 1978), although preferences do not always correlate positively with offspring performance (e.g. Valladares and Lawton 1991; Clark et al. 2011), because other ecological, behavioural and physiological factors additionally influence host choice and host use (Jaenike 1990). While *D. suzukii* deposited its eggs on fruits from 16 different plant families in our study, we do not expect its offspring to perform equally well across the host spectrum, because preferences of insects with a broader host plant spectrum are less strongly associated with better offspring performance than insects specialised on plants within a certain family (Gripenberg et al. 2010). However, invasions are biologically unusual situations, and *D. suzukii* cannot be expected to show strong adaptive patterns of host use yet, as it was only recently introduced. Thus, some differences in larval performance might be expected and have also been shown between many non-crop hosts of *D. suzukii* (e.g. Poyet et al. 2015; Kenis et al. 2016; Olazcuaga et al. 2019).

Plant communities differ floristically and phytophysiognomically (Braun-Blanquet 1932) because species traits and an interacting milieu affect performance across environmental gradients such as temperature, moisture and soil chemistry (Mcgill et al. 2006). Therefore, the abundance and availability of host plants among forest communities must also differ. In our NMDS of available fruits of potential host plants in the plots, the forest communities were clustered. This finding and our model results indicated that the availability of different plant species determines the use of the host plants, as the forest community affected the attack rate. In addition to the diversity of host plants in an area, other associational effects, such as the density and frequency of neighbouring host plants, can influence the likelihood of a particular plant being used as a host, as can the density of the particular host plant (Underwood et al. 2014). As stated by the resource concentration hypothesis (Root 1973), a high density of host plants may increase the likelihood that the fly will find the fruits and remain on the host plant. This was supported by our results, as the number of ripe fruits present in our plots strongly affected the attack rate. Furthermore, since optimal oviposition behaviour depends not only on the suitability of the substrate, but also on the probability of finding a more suitable host in the time available (Jaenike 1978), adherence to a particular host plant species might be advantageous. A large amount of fruits may be more obvious to *D. suzukii* and thus increase the attack rate. A strong preference for more apparent resources in complex environments has also been shown for *D. melanogaster* (Verschut et al. 2016).

Our results further show that a longer fruit duration of fruits present increased the attack rate. Plants with more fruits, and especially plants such as brambles that produce ripe fruits over a long period, ensure continuous availability of resources without the need to search for new hosts, thus promoting the presence of overlapping generations of *D. suzukii* on the same host, further increasing attack rate. In Switzerland, between four and eight generations of *D. suzukii* per year are expected (Wiman et al. 2014). In addition, prior experience with olfactory and visual cues can enhance host location (Silva and Clarke 2020). Furthermore, a later ripening date was related to a higher attack rate, which may occur when the peak of abundance of *D. suzukii* and the period of fruit maturity coincide. The predominant maturity status of the plants in the plots also affected the attack rate. The more advanced the maturity, the greater the attack rate of the ripe fruits investigated. An advanced maturity status attracts more flies (Keesey et al. 2015), but also indicates overlapping generations on the same plant.

Adult trapping revealed *Drosophila suzukii* as a dominant species

The high fruit attack rate observed in our study was also reflected in a high abundance and dominance of *D. suzukii* in trap catches, independent of forest community and elevation. This indicates the broad environmental tolerance of the fly. However, frequent occurrence in mountainous regions does not necessarily mean that *D. suzukii* inhabits these regions year-round, as studies from Japan and the Italian Alps suggest that the fly exhibits extensive movements between low and high elevations (Mitsui et al. 2010; Tait et al. 2018) and is capable of travelling distances of up to 9 km (Tait et al. 2018). However, it can be assumed

that the fly can survive the winter at these sites, as *D. suzukii* is firmly established on the island of Hokkaido in Japan, where winters average -4 to -12 °C (Kimura 2004).

Although our forest communities differed significantly in host composition, there were no differences in the proportion of *D. suzukii* between the forest communities. *Drosophila suzukii* accounted for 86% of the drosophilids caught during our study period. Recent snapshot studies of trap catches in forests also showed a high proportion of *D. suzukii*. In a survey in native riparian and non-riparian chestnut forest patches in northwestern Spain in August, *D. suzukii* accounted for 30% and 27% of the drosophilids caught in beer traps (Maceda-Veiga et al. 2021). Kremmer et al. (2017) reported 56% *D. suzukii* of the summed trap catches across natural habitat and crops in February (baited with apple cider vinegar). Studies in agricultural areas similar to our sites in terms of trapping period, climate and sampling method do not confirm *D. suzukii* as the most abundant taxon: *D. suzukii* accounted for 7% in Apulia, Italy (Antonacci et al. 2017), 11% in Kansas, USA (but baited with mashed banana; Gleason et al. 2019), and 18% in Washington, USA) (but some unmanaged habitats included; Bahder et al. 2016). Several factors could have caused these differences. First, agricultural and forested areas differ in biotic and abiotic factors, and species composition thus also differs (Burla and Bächli 1991). The assemblages of drosophilids, excluding *D. suzukii*, trapped in these agricultural areas were dominated by domestic species (distributed worldwide), while native species dominated in our area. Similarly, the occurrence of controphics, such as other insects, vertebrates or mould, and the occurrence of predators differ. Second, competition between *D. suzukii* and native species might be different in the two habitat types, due to differences in the occurrence of ecologically related species and available resources. Third, different measures taken to control the fly in agricultural fields might have reduced the populations of *D. suzukii*. However, these measures usually also affect other drosophilids. Fourth, forests can be expected to be the preferred habitat of *D. suzukii*, resulting in high overall proportions. Numerous forest fruits are suitable for the development of *D. suzukii*, due to its wide host niche (Little et al. 2020), and forests may offer preferred climatic conditions for *D. suzukii*, especially higher humidity (Hamby et al. 2016; Tochen et al. 2016; Eben et al. 2018).

Unlike in our study, surveys of drosophilid assemblages from trapping studies in various habitats in Asia, where *D. suzukii* is native or has been established for many years, do not show *D. suzukii* to be a dominant species: its proportion in relation to other drosophilids is reported to be up to 0.02 (e.g. Kaneko and Tokumitsu 1969; Toda 1992; Hirai et al. 2000; Guruprasad et al. 2010) and reached a value of 0.05 in a study in Korea (Lee 1964). Parasitisation is, however, an important mortality factor for drosophilid larvae (Janssen et al. 1988; Fleury et al. 2009), and the abundance of potential hosts is one factor determining the evolution of parasitoid host use (Novković et al. 2012). The degree of parasitisation is much higher in native populations (Torchin et al. 2003). The expected lower parasitisation of *D. suzukii* could be one of the main factors contributing to its frequent occurrence outside its native range, as escape from natural enemies can explain the success of introduced species (Keane 2002; Shea 2002).

Impact on native drosophilids

Our trap catches revealed that the abundance of *D. suzukii* was significantly negatively associated with the abundance of other drosophilids. Its dominance indicates a superiority over the native species. The heavily sclerotised, serrated ovipositor serves as a competitively unique trait (Karageorgi et al. 2017; Silva-Soares et al. 2017). Besides the capability of using a wider range of substrates for oviposition, we propose that the broader temporal niche of resource use is a competitive advantage. *Drosophila suzukii* can use substrates for oviposition earlier than other drosophilid species. Consequently, oviposition by *D. suzukii* induces substrate decay, rendering the substrate time-limited for the larval development of other fruit pulp consumers. While ripe fruits are inherently a temporally limited resource, this limitation is probably enhanced in forests compared with agricultural areas, because the fleshy fruits of wild plants are usually much smaller than those of cultivated plants. However, oviposition of *D. suzukii* on long-lasting larval habitats such as cultivated fruits may allow further use by other drosophilids (Rombaut et al. 2017; but see Entling and Hoffmann 2020).

Furthermore, *D. suzukii* may outcompete native drosophilids, due to different development times. This might lead to different competitiveness in forests and agriculture. Cold adaptation in *Drosophila* to colonise temperate climates appears to be associated with longer development times (Santos et al. 2006; Kinzner et al. 2018), and cosmopolitan human commensals (“domestic species”) such as *D. melanogaster*, which originated in central equatorial Africa (Lachaise and Silvain 2004), have comparatively short development times (Markow and O’Grady 2005a). In laboratory experiments, it has been found that the presence of *D. melanogaster* on the substrate significantly reduced adult *D. suzukii* emergence, reflecting a difference in minimum development time (7 days for *D. melanogaster* and 11 days for *D. suzukii* at 25 °C; (Dancau et al. 2017; Shaw et al. 2018). Presumably, the presence of *D. suzukii* reduces native drosophilid emergence as well. Coexistence of drosophilids across food-limited resource patches is facilitated by aggregation over patches (eggs in clutches of more than one egg, as well as non-random distributions of ovipositing females), which creates partial refuges and allows inferior species to exist (Rosewell et al. 1990; Jaenike and James 1991; Sevenster and Alphen 1996; Rohlf and Hoffmeister 2003). In addition, parasitism facilitates the coexistence of drosophilid species (Mitsui and Kimura 2000a). However, as an invasive species, *D. suzukii* is expected to be exposed to lower pressure from parasitoids (Torchin et al. 2003), resulting in larger populations. Unlike other drosophilids, *D. suzukii* has been shown not to aggregate over patches (Mitsui et al. 2006). Therefore, we suggest that it may exclude and outcompete other drosophilids in the exploitation of resource patches, which is supported by our results: higher abundance of *D. suzukii* was associated with lower abundance of other drosophilids.

Potential impact on host plants

Piercing of the skin of undamaged fruit by *D. suzukii* provides an entry point for infestation by pathogens, and inoculated microbes can act as a jump-start for decay.

Microbes that infect fruits have been hypothesised to make fruits unattractive to vertebrate frugivores that potentially compete for fruit pulp (Janzen 1977; Ruxton et al. 2014). Just as humans respond to infested fruit with rejection, birds have been shown to prefer intact fruit to infested fruit (e.g. Manzur and Courtney 1984; Jordano 1987; Borowicz 1988; Buchholz and Levey 1990; Cipollini and Stiles 1993; Traveset et al. 1995; but see Valburg 1992). Attacks by *D. suzukii* alter the attractiveness of the fruit because it changes the chemical composition and visual cues, such as colour, shape and reflective patterns. Since the choice of fruits by birds depends on visual perception (Schaefer and Ruxton 2011), we assume that attacks on fruits by *D. suzukii* could reduce the attractiveness for birds, resulting in reduced seed dispersal. This is because many host plants of *D. suzukii* rely especially on frugivorous birds for dispersal (Garcia et al. 2010). Negative effects on seeds are unexpected because attacks occur after seed set.

In the field, when we observed symptoms of fruit decay after *D. suzukii* attacks, we noticed that fruit decay progressed at very different rates among plant species, which may be due to their different compounds and compositions. For example, the bright red fruits of *Lonicera alpigena* were found to change into dry, brown fruit mummies (rotten fruits) within a short time, while fruits of *Prunus padus* had hardly any symptoms of decay and hung intact on the bushes for more than 40 days without decaying. On *Prunus padus*, a significant reduction in the number of emerged *D. suzukii* adults and a significant delay in the larval-pupal development time was detected when compared with *Prunus avium* (Alhmedi et al. 2019), where oviposition by *D. suzukii* is known to trigger microbial development (Ayyanath et al. 2018). Because larval development depends on the development of microbes (Sang 1956; Schwarz et al. 2014; Hamby and Becher 2016), decay within a short time indicates a strong response of microbial growth and development and better host suitability to *D. suzukii* offspring development. Among the fruit traits we studied, we found that the pulp sugar content determined the severity of the symptoms of decay after an attack by *D. suzukii*. The rapid decomposition of simple carbohydrates leads to rapid microbial growth (Zhao et al. 2020), explaining why more severe symptoms of decay were evident at higher sugar levels. Nutrient levels deplete over time as microbes and larvae consume the resources, so rapid fruit decay after an attack by *D. suzukii* means a loss of resources for other frugivores.

Conclusion

Almost half of the 39 studied forest plant species showed attack rates by *D. suzukii* of > 50%, with a high percentage showing severe symptoms of fruit decay after egg deposition. This may lead to reduced fruit consumption by vertebrate seed dispersers. *Drosophila suzukii* is a damaging agent for plants, and if the fly reproduces in large numbers, dispersal agents and host plants may both suffer. Besides the direct effect of parasitism of forest fruits by *D. suzukii*, leading to competition with other frugivorous species, the indirect effect of disrupting seed-dispersal mutualisms can have far-reaching consequences for ecosystems. With ongoing climate change, these potentially

severe ecological impacts might be amplified in temperate forests, as higher average and winter temperatures will most likely lead to shorter generation times and lower winter mortality, which will eventually further increase the pressure on forest fruits and the competitiveness of *D. suzukii* over native drosophilids.

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Supplementary material I

Tables S1, Figure S1

Authors: Irene Bühlmann, Martin M. Gossner

Data type: Docx file.

Explanation note: Supplementary information on potential host plant species of *Drosophila suzukii* in the study plots and Boxplots of proportions of fruits attacked by *Drosophila suzukii* per species, including fruits outside the plots, separated by forest community.

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Link: <https://doi.org/10.3897/neobiota.77.87319.suppl1>

Supplementary material 2

Table S2

Authors: Irene Bühlmann, Martin M. Gossner

Data type: Xlsx file.

Explanation note: Abundances of sampled insect taxa, based on catches from cup traps with 3 mm diameter entry holes baited with apple cider vinegar in different forest communities.

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Link: <https://doi.org/10.3897/neobiota.77.87319.suppl2>

Integrating expert knowledge at regional and national scales improves impact assessments of non-native species

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Abstract

Knowledge of the impacts of invasive species is important for their management, prioritisation of control efforts and policy decisions. We investigated how British and Irish botanical experts assessed impacts at smaller scales in areas where they were familiar with the flora. Experts were asked to select the 10 plants that they considered were having the largest impacts in their areas. They also scored the local impacts of 10 plant species that had been previously scored to have the highest impacts at the scale of Great Britain. Impacts were scored using the modified classification scheme of the EICAT framework (Environmental Impact Classification for Alien Taxa). A total of 782 species/score combinations were received, of which 123 were non-native plants in 86 recording areas. *Impatiens glandulifera*, *Reynoutria japonica* and *Rhododendron ponticum* were the three species considered to have the highest impacts across all regions. Four of the species included in the list of the 10 highest impact species in Great Britain were also in the top 10 of species reported in our study. Species in the higher impact categories had, on average, a wider distribution than species with impacts categorised at lower levels. The main habitat types affected were woodlands, followed by linear/boundary features and freshwater habitats. Thirty-nine native plant species were reported to be negatively affected. In comparison to the overall non-native flora of Britain and Ireland, the lifeform spectrum of the species reported was significantly different, with higher percentages of aquatic plants and trees, but a lower proportion of annuals. The study demonstrates the value of local knowledge and expertise in identifying invasive species with negative impacts on the environment. Local knowledge is useful to both confirm national assessments and to identify species and impacts on native species and habitats that may not have gained national attention.

Keywords

Alien plants, biological recording, Europe, habitat impacts, impact, local knowledge, scoring

Introduction

Invasive non-native species continue to be introduced, spread and cause environmental and socio-economic impacts globally (Diagne et al. 2021; Seebens et al. 2021). They are one of the main drivers of global biodiversity decline (IPBES 2019; Dueñas et al. 2021) and species extinctions (Bellard et al. 2016). There is, therefore, a need for consistent appraisals of impacts to support risk assessments, decisions on invasive species management, prioritisation of control efforts and policy-making. Impact assessments of invasive species play an important part in risk assessments and support policy decisions based on them (Leung et al. 2012). For example, one key measure of the EU Regulation (No 1143/2014) on invasive species is the listing of species of Union concern, which are species that *‘based on available scientific evidence, are likely to have a significant adverse impact on biodiversity or the related ecosystem services and may also have an adverse impact on human health or the economy’* (§4c, EU 2014).

To support these policy and management decisions and to advance the understanding of how invasive species’ impacts are assessed across taxa and regions, frameworks and methodologies for delivering this evidence in a consistent and comparable way have been developed (Bartz and Kowarik 2019; González-Moreno et al. 2019; Vilà et al. 2019). In 2020, the IUCN adopted the EICAT framework (Environmental Impact Classification of Alien Taxa) developed by Hawkins et al. (2015) as its global standard for impact assessments (IUCN 2020; Volery et al. 2020). The EICAT methodology has been applied at the global level for the assessment of introduced birds (Evans et al. 2016), bamboos (Canavan et al. 2019), reptiles and amphibians (Kraus 2015) and ungulates (Volery et al. 2021), amongst others. At regional levels, it has been used for non-native mammals in Cuba (Borrito-Páez and Mancina 2017) and invasive grasses in South Africa (Visser et al. 2017).

A modified version of the EICAT framework was first applied in 2016 for a national impact assessment applied across taxon groups (Booy 2019). Specifically, it was used to assess a selection of 238 non-native species in Great Britain (GB), which had been identified from a longer list of 1,954 established non-natives and considered to have more than minimal impacts, based on information in the GB Non-Native Species Information Portal database (Booy 2019). The modified EICAT scoring was carried out by experts using published evidence, anecdotal knowledge and their own field experience, followed by a consensus workshop (Booy 2019). For non-native plants, a group of seven experts (including authors KDS and KJW) assessed 122 species that were identified in the pre-screening process. All plants were assigned an impact rating, 10 of which were rated in the top two EICAT impact levels (major and massive). This GB impact assessment process illustrates how impact assessments rely on evidence mostly generated in field or experimental studies at local levels (Vilà et al. 2019), before being evaluated and

extrapolated in expert assessments to national levels. However, as impacts are context-dependent (Bartz and Kowarik 2019), this approach may overestimate impacts at larger scales. It could also result in species with major and/or emerging local impacts not being considered as important species at larger scales. Consequently, species with more local impacts could be missed from policy frameworks and their management may not be supported sufficiently. Similarly, experts involved in scoring at the national scale could over- or underestimate the impacts of species. For example, where available evidence is only locally available, anecdotal or from other territories or in the absence of impact data, they may rely solely on distribution data which may not always relate to important local-scale impacts (Pearman and Walker 2009). The 2016 GB scoring, therefore, offered an opportunity to test the results of the national assessment with a group of local experts, i.e. regional volunteer expert botanical recorders of the Botanical Society of Britain and Ireland (BSBI). In contrast to experts evaluating published evidence and data, these botanical recorders are more likely to base their impact judgements on their field experience of the region in which they work, their knowledge of local occurrences of native and non-native plants from recording activities (Pescott et al. 2015) and often, their experience of change in species distributions from their long-term involvement in these local activities (Preston 2003; Walker 2003). These local botanical experts are, therefore, also uniquely placed to observe habitats and native species affected by non-native species invasions.

Different species traits may explain the success of species at different stages of the invasion process and this has been investigated for different stages of the plant invasion process (Dehnen-Schmutz et al. 2007; Pyšek and Richardson 2007). Few studies have investigated how species traits influence invasive species' impacts; however, amongst investigated traits, lifeform has already been shown to be important (Pyšek et al. 2012; Ni et al. 2021). The aim of our study was, therefore, to investigate which non-native plants are currently perceived to have the highest impacts at regional scales, as well as across Britain and Ireland, based on the knowledge of local experts. We then use these data to analyse if there are differences in these perceptions between regions and how the impacts of the 10 non-native plant species that were previously assessed to have the highest impacts in the 2016 GB assessment (Booy 2019) were assessed at regional levels by these local experts. Furthermore, we used species distribution data and plant lifeform characteristics to investigate the relationships between local impact assessment scores and range size and between assessment score and lifeform. Finally, we explore which broad habitats and native species were most affected by the invasive non-native species identified by local experts.

Methods

Study area and participants

The study was conducted at the regional scale in 152 recording units, known as 'Watsonian vice-counties', across the whole of Britain and Ireland (Fig. 1). These vice-counties (VC) were devised in 1852 with the aim of creating a series of stable geographical

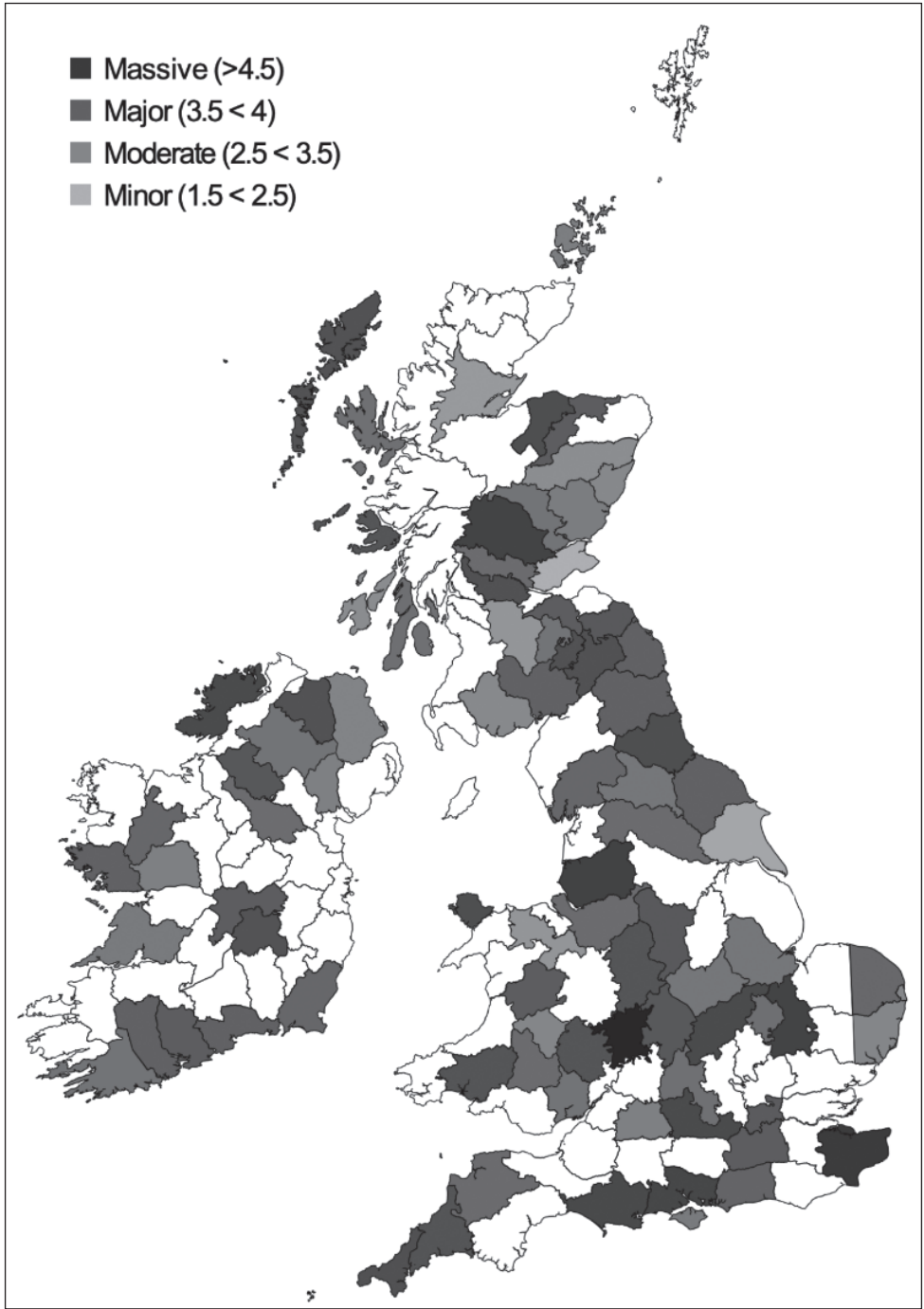


Figure 1. Map showing vice-county boundaries in Britain and Ireland. Vice-counties participating in the study are coloured in a grey-scale representing the average impact score of the species reported.

units for recording natural history observations and continue to be used to this day by the Botanical Society of Britain and Ireland (BSBI) and other societies (Preston et al. 2002). These vice-counties have an average size of about 2000 km², with a range of 207 to 4857 km² (Fig. 1).

The BSBI is the backbone of botanical recording in Britain and Ireland (Pescott et al. 2015) and appoints volunteer vice-county recorders to both collect and collate botanical records for their areas and to submit them to the BSBI centrally (<https://database.bsbi.org/maps/>). Vice-county recorders, therefore, have an unrivalled knowledge of the plant species, both native and non-native, that occur at a local level, including their distribution, abundance, habitats and regeneration, while also being aware of impacts on other plant species and ecosystem processes. Vice-county recorders existed long before the term ‘citizen science’ was used in Britain; however, because of their voluntary role, they would now also be considered citizen scientists (Pescott et al. 2015). Considering their expertise and their focused recording efforts (systematic recording of all plant species in contrast to unstructured or opportunistic recording) and following the classification of citizen scientists suggested by Pocock et al. (2015), these vice-county recorders would be classified as volunteer regional experts.

Survey design

An online questionnaire was designed using the ‘Online surveys’ platform (www.onlinesurveys.ac.uk). The survey link was distributed to all BSBI vice-county recorders in March 2019 and the survey was closed at the end of May 2019. The questionnaire (see Suppl. material 1, Part 1) consisted of a short introduction, followed by an explanation of the criteria for scoring species’ impacts and the respondents’ confidence (Table 1). Participants were asked to name the 10 plant species that they considered were having the highest environmental impacts in their vice-counties, based on the modified EICAT scoring criteria (Table 1). Respondents were also asked to name the species with the highest impacts first, thus creating a list ranked by their perception of local impact. Each species’ impact score was assigned to a broad habitat type from a list provided, corresponding to the UK Biodiversity Action Plan habitat descriptions (<http://jncc.defra.gov.uk/page-5706>). In the second part of the survey, participants based in Great Britain were presented with the list of the top 10 highest ranked plant species from the 2016 GB national-level impact workshop (Booy 2019) and asked to score the impacts of these species in their vice-county using the same criteria, but only if they had not already included them in their top 10 scoring list in the previous section of the survey. Vice-county recorders from Ireland did not participate in this part of the survey, because the 2016 impact scoring was conducted for Great Britain only. In both sections, respondents could leave comments for individual species and were asked to do so, in particular, if they were aware of impacts in habitats considered as priority in the UK Biodiversity Action Plan or invasives that were known to have caused the local extinction of any native species. The questionnaire was approved through Coventry University’s ethical approval procedure (Ethic no: P87103).

Table 1. Definition of impact categories and confidence scores as presented to the participants in the survey in Great Britain and Ireland. Modified from the EICAT framework (Hawkins et al. 2015).

Impact categories	Confidence Score
1. Minimal Concern ... unlikely to have caused deleterious impacts on the native biota or abiotic environment.	High ... approx. 90% chance of assessment being correct, i.e. direct observational evidence by the assessor or there are reliable/good quality data sources on impacts of the taxa in the VC.
2. Minor ... causes reductions in the fitness of individuals in the native biota, but no declines in native population sizes and has no impacts that would cause it to be classified in a higher impact category.	Medium ... defined as 65–75% chance of the assessor score being correct, i.e. there is some direct observational evidence to support the assessment, but some information is inferred.
3. Moderate ... causes declines in the population size of native species, but no changes to the structure of communities or to the abiotic or biotic composition of ecosystems and has no impacts that would cause it to be classified in a higher impact category.	Low ...only about 35% chance of being correct, i.e. no direct observational evidence or reliable data sources to support the assessment, for example, only inferred data have been used as supporting evidence.
4. Major ... causes the local or population extinction of at least one native species and/or leads to substantial, but reversible changes in the structure of communities and the abiotic or biotic composition of ecosystems and has no impacts that cause it to be classified in the MV impact category.	
5. Massive ... leads to the replacement and local extinction of native species and produces irreversible changes in the structure of communities and the abiotic or biotic composition of ecosystems.	

Impact scoring framework

We used the modified scoring categories that were applied in the 2016 national GB assessment (Booy 2019), based on the EICAT methodology (Hawkins et al. 2015). Impacts were scored, based on the five categories namely ‘massive’, ‘major’, ‘moderate’, ‘minor’ and ‘minimal’ concern (Table 1). The modification of the EICAT categories in the GB assessment involved a change in the definition of the ‘major’ category, which was changed to specify that a species extinction (national or local) or a substantial, but reversible change in affected ecosystems needs to be observed, in contrast to the EICAT scheme, where both conditions need to be fulfilled (extinction and reversible change). For each species’ score, respondents were also asked to score their confidence following the definitions given in the survey (Table 1) and based on the GB assessment’s definition. In contrast to the EICAT framework and the GB assessment, which distinguished current and maximum impacts that could be reached if a species would occupy all suitable habitats, respondents in our survey were only asked to score the current impacts.

Data analysis

To identify the highest scoring species and to compare impact scores between species and vice-county recorders, we assigned each impact category an integer value, ranging from 1 for the ‘minimal’ category to 5 for the ‘massive’ category. We also compared the rank order in which respondents had reported the 10 species for their vice-county as they were asked to report species in the order of perceived impact magnitude from highest to lowest.

We derived the list of highest scoring species for the whole region using three complementary approaches. First, we ranked species by the number of respondents who had included them in their list of 10. Second, we calculated an average impact score for each species, both for the whole sample (with an impact score of zero for vice-counties in which the species was not included in the top 10), as well as the average for only those vice-counties that included the species in their top list. Third, we added the rank scores for each species (where listed by a respondent) and ranked the overall species list by these sums. To analyse the consistency in the scoring, we followed the methodology used by González-Moreno et al. (2019) and calculated a coefficient of variation (CV) for all species that had received at least four scores. We also used the CV to compare the variation in scoring between respondents (i.e. how they used the scale of impact categories across the 10 species).

Species' current distribution data were extracted from the BSBI's Distribution Database (<https://database.bsbi.org/>) at a 2×2 km gridded resolution in March 2022. As the size of the vice-counties varies, we used the percentage of total 2×2 km grid cells occupied per vice-county. For nine reports of species with no records in particular counties, we used an arbitrary value of one grid cell. Nomenclature and common names for vascular plants followed Stace (2019), while species' native or non-native status was based on Preston et al. (2002). In cases where species that are native in some parts of Britain or Ireland were reported, we confirmed the status in the vice-county from where it was reported as non-native. Data on the Raunkiaer lifeform of the reported species was derived from ALIENATT, an unpublished BSBI compilation which is also the basis for this information in Henniges et al. (2022). A list of the 1,690 non-native plants confirmed in Britain and Ireland was extracted from Henniges et al. (2022). This list was used to compare the lifeform of plants (chamaephyte, geophyte, hemcryptophyte, helophyte, hydrophyte, phanerophyte, therophyte) reported in the survey using a two-sample z-test of proportions and *post hoc* row-wise Fisher tests with *p*-values adjusted for pairwise comparisons between proportions of single categories. Species reported in the survey that are native in parts of Britain and Ireland and non-native in others were excluded from this comparison (i.e. *Clematis vitalba*, *Fagus sylvatica*, *Nymphoides peltata*, *Papaver cambricum*, *Pinus sylvestris*, *Ranunculus* subgenus *Batrachium*, *Stratiotes aloides*, *Symphytum tuberosum* and *Typha latifolia*).

Where respondents had not assigned a main broad habitat type, we used the comments provided by them to assign a main habitat. Out of 75 species (9.6% of all species reported) with no habitat assignment, we allocated 61 species to habitats including the two additional habitat categories 'urban' and 'brownfield', that were not included as an option in the survey design. The remaining 14 species which could not be allocated or where the respondent indicated the species could not be assigned to a main habitat type, were categorised as 'other'. The International Union for the Conservation of Nature's (IUCN) Red List status of species reported to be threatened by invasive plants was checked for records in GB and Ireland separately in the respective lists as compiled in Wyse Jackson et al. (2016). We conducted all analyses in the R environment, version 4.0.3 (R Core Team 2019), including the bipartite (Dormann et al. 2008) and vegan packages (Oksanen et al. 2019).

Data availability statement

The data are made available in Suppl. material 2.

Results

Results for the whole region

We received responses from 86 vice-counties (a return rate of 57%), reporting a total of 123 species from 782 vice-county observations. While most recorders reported 10 species, some reported fewer and, for one vice-county, eleven species were named, giving a range of one to eleven species and an average of 9.1 (s.d. = 1.96) per vice-county. There were more than 100 observations of ‘massive’ impact species; however, the majority of reports related to evaluations of ‘major’ or ‘moderate’ impacts (Fig. 2).

The mean coefficient of variation (CV) of impact scores per species named by at least four recorders was 32% (s.d. = 9), with a range from 17% for *Prunus laurocerasus* from 20 reports to 51% for *Gunnera tinctoria* from seven reports. The CV across all recorders was 26%, based on the average score given to all species scored per vice-county of 3.3 (s.d. = 0.85). The average scores per vice-county can be seen in Fig. 1. *Impatiens glandulifera*, *Reynoutria japonica* and *Rhododendron ponticum* were the three species considered to have the highest impacts across all vice-counties by

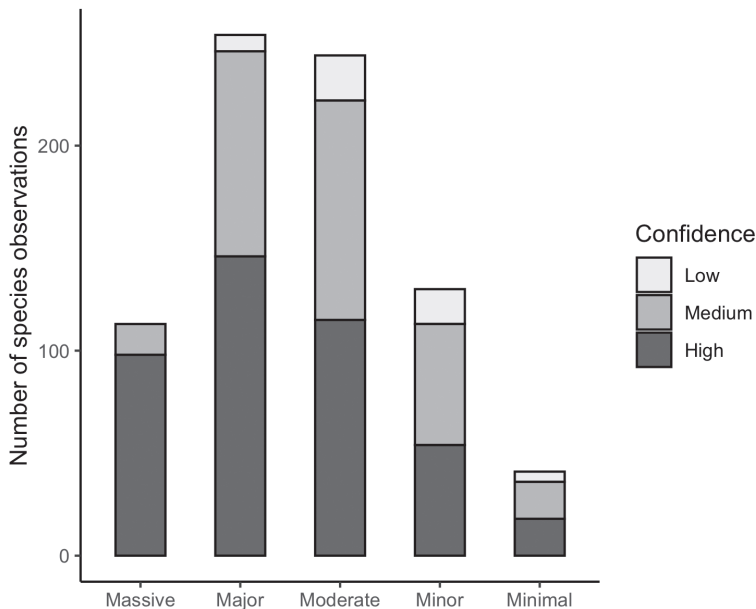


Figure 2. Frequency of impact and confidence scores received from vice-county recorders. The number of observations relates to 123 different species reported.

Table 2. Most frequently reported species with impacts ordered by the number of vice-counties (VCs) that included the species in their top 10 (N), this number as a percentage of all vice-counties (%), the mean impact score from vice-counties that reported this species (Mean VCs present), the mean impact score for all vice-counties (Mean overall), rank position when all rank scores were summed up (Rank-sum rank) and the coefficient of variation for all scores received for each species (CV).

Species	N	%	Mean VCs present	Mean overall	Rank-sum rank	CV
<i>Impatiens glandulifera</i>	63	73	3.62	2.65	1	31.94
<i>Reynoutria japonica</i>	60	70	3.28	2.29	2	30.76
<i>Rhododendron ponticum</i>	58	67	3.81	2.57	3	28.44
<i>Crassula helmsii</i>	36	42	4.00	1.67	4	21.55
<i>Heracleum mantegazzianum</i>	36	42	3.33	1.40	5	28.69
<i>Picea sitchensis</i>	23	27	3.70	0.99	6	28.77
<i>Cotoneaster integrifolius</i>	20	23	3.75	0.87	10	20.97
<i>Lamium galeobdolon</i> ssp. <i>argentatum</i>	20	23	3.25	0.76	13	28.01
<i>Prunus laurocerasus</i>	20	23	3.60	0.84	7	16.62
<i>Acer pseudoplatanus</i>	19	22	3.58	0.79	8	25.19
<i>Allium paradoxum</i>	17	20	3.65	0.72	11	33.50
<i>Hyacinthoides</i> × <i>massartiana</i>	17	20	2.82	0.56	14	28.65
<i>Lysichiton americanus</i>	16	19	2.69	0.50	17	50.32
<i>Symphoricarpos albus</i>	16	19	3.06	0.57	16	18.73
<i>Hydrocotyle ranunculoides</i>	15	17	3.73	0.65	9	32.75
<i>Myriophyllum aquaticum</i>	15	17	3.00	0.52	15	43.64
<i>Elodea canadensis</i>	14	16	3.64	0.59	12	25.50
<i>Azolla filiculoides</i>	13	15	3.08	0.47	18	31.01
<i>Petasites fragrans</i>	13	15	3.46	0.52	19	27.95
<i>Crocodymia</i> × <i>crocodymiflora</i>	12	14	2.58	0.36	22	30.70
<i>Elodea nuttallii</i>	12	14	3.33	0.47	23	32.19
<i>Buddleja davidii</i>	11	13	3.27	0.42	21	33.72
<i>Rubus spectabilis</i>	11	13	3.55	0.45	20	26.35

frequency, mean overall impact score and ranking, followed by *Crassula helmsii*, *Heracleum mantegazzianum* and *Picea sitchensis* in exactly the same positions by each measure (Table 2).

In comparison to the GB assessment, we found that four of the top 10 species from that assessment were also in our top 10 list (Table 3) and a further three included in our list of species reported from more than 10 vice-counties (Table 2). Just one species, *R. japonica*, was present in all British vice-counties; however, only 68% of respondents included the species in their county's top 10. Similar high presences in the top 10 list of vice-counties were only achieved by *R. ponticum* and *C. helmsii* (Table 2). Species where impacts are mainly recorded in coastal habitats, i.e. *Carpobrotus edulis*, *Cotoneaster integrifolius* and *Spartina anglica*, were only in the top 10 of a few vice-counties as these habitats are not available in inland areas. The top 10 GB list included one moss, *Campylopus introflexus*; however, the BSBI and their recorders do not record bryophytes and this was confirmed by 20 of the respondents who did not comment on the species' presence in their county, with many of them indicating that moss species are outside of their remit.

Table 3. Assessment of the top 10 GB species (Booy 2019) in the 68 vice-counties located in Great Britain. Species presence (‘In top 10’) in the list of 10 highest impact plants in the respective county, presence in county, but not in top 10 (‘Present’), species reported as not present (‘Not present’) and number of respondents not answering the question (NA).

Species	In top 10	Present	Not present	NA
<i>Azolla filiculoides</i>	10	32	25	1
<i>Campylopus introflexus</i>	4	23	21	20
<i>Carpobrotus edulis</i>	2	10	53	3
<i>Cotoneaster integrifolius</i> *	9	38	15	6
<i>Crassula helmsii</i> *	39	19	9	1
<i>Reynoutria japonica</i> *	46	18	0	4
<i>Hydrocotyle ranunculoides</i>	15	10	39	4
<i>Myriophyllum aquaticum</i>	12	22	30	4
<i>Rhododendron ponticum</i> *	45	20	1	2
<i>Spartina anglica</i>	6	19	41	2

* indicates species is also in the overall top 10 list for all vice-counties.

Regional differences

Regions differed in the number of species reported (Suppl. material 1: Table S1). Looking at the frequency of individual species reported, for each region there were species in the list of species more frequently reported that were not in the overall high frequency list (Table 2). For Ireland, *Allium triquetrum* (5 counties) and *Pinus contorta* (5) were in the top 10 list of species, *Claytonia sibirica* (3) and *Tolmiea menziesii* (3) were in the top 10 list of species in the Scottish Highlands and Islands, with *T. menziesii* (5) also frequent in the Scottish Lowlands. *C. edulis* was in the top list of two of the five vice-counties participating in the region of Southwest England.

Raunkiaer plant lifeform

Most observations of species with impacts were related to phanerophytes, with more than half of these impacts considered to be ‘massive’ or ‘major’ (Fig. 3A). There was a significant difference (probability test of proportion: chi-squared = 51.5, d.f. = 5, *p*-value < 0.001) in the lifeform spectrum (Fig. 3B) of the species reported in the survey (excluding species with native occurrences in some parts of the study area) to all non-native plants included in the species list of the British and Irish flora (Henniges et al. 2022). The *post hoc* test of pairwise comparisons between proportions of lifeform categories found significant differences for the combined category of helophytes and hydrophytes (*p* < 0.001), which contribute 1.8% of the non-native flora of the British Isles, but 10.6% of the species reported to have impacts. Focusing more specifically on aquatic plants, all but three of the 14 non-native species recorded in the flora of Britain and Ireland were reported amongst the non-native species with impacts. Therophytes were the second group with significant differences in the *post hoc* test (*p* < 0.001) contributing 28% of all non-natives, but just 10% in our study (for all pairwise post-hoc comparisons, see Suppl. material 1: Table S2).

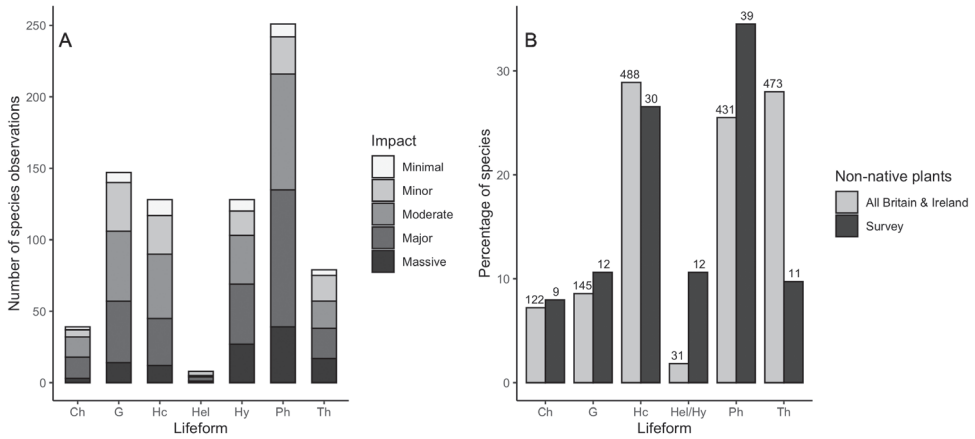


Figure 3. A number of observations and impact categories of 113 higher non-native plants reported in the survey and **B** lifeform comparison of the non-native plants with reported impacts to all 1,690 non-native plants recorded in the British and Irish flora (Henniges et al. 2022). Bars show the percentage of each sample for each lifeform, labels the number of species in each category (data for the survey in B exclude nine species that are native in some parts of the study area). Raunkiaer plant lifeforms: chamaephyte (Ch), geophyte (G), hemcryptophyte (Hc), helophyte (Hel), hydrophyte (Hy), phanerophyte (Ph), therophyte (Th).

Impacts by broad habitats and native species affected

Respondents reported the main broad habitat affected for each species when scoring the species' impacts. The highest number of species was reported for broadleaved/mixed woodlands, followed by boundary/linear features, rivers and streams and standing open waters/canals (Suppl. material 1: Table S3). Looking more closely at which species are reported from which habitats (bipartite graph Suppl. material 3: Fig. S1), we find some species predominantly reported from single broad habitat types, for example, *P. laurocerasus*, *Rubus spectabilis* and *Lamium galeobdolon* subsp. *argenteum* for broadleaved/mixed woodlands or *I. glandulifera* for rivers and streams. In contrast, other species are reported to have impacts in several habitats, most notably *R. japonica*, which was found along rivers and streams, broadleaved/mixed woodlands, urban/brownfield, inland rock, littoral habitats and boundary/linear feature habitats. In boundary/linear feature habitats, we found the greatest mixture of species that are also found in other habitat types, reflecting the diverse range of habitat conditions in this category (Suppl. material 3: Fig. S1).

Additional comments provided by respondents gave further insight into individual cases of habitat impacts. For example, one respondent reported the massive impact of *Quercus ilex* on calcareous grassland that had resulted in the “complete loss of chalk grassland”. A respondent from Ireland reported *R. spectabilis* with massive impacts in broadleaved woodlands commenting “by far the single biggest threat to what remains of our broadleaved woodlands. Have seen woodland where this is the only ground-level species”. Further priority habitats mentioned specifically in comments include

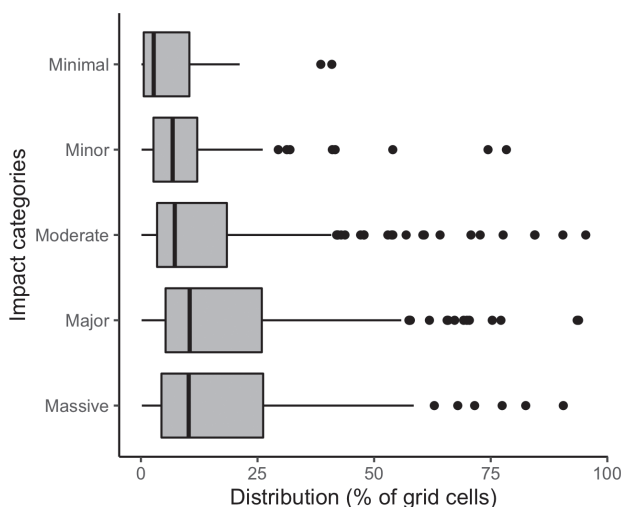


Figure 4. Distribution of reported invasive plants in vice-counties by impact category. The 2 km-scale range size of species is shown as the percentage of the total number of 2 km grid cells per vice-county.

oligotrophic lakes, limestone pavements, hedgerows, oak woodlands, blanket bogs and coastal sand dunes.

Respondents also named 39 native plant species that were particularly negatively affected by the invasive plant they listed, including several species listed in the vascular plant Red Lists for Britain and Ireland, respectively (Table 4). Three species are classified as ‘Vulnerable’: (1) the liverwort *Southbya nigrella*, which reaches its northern distributional range on calcareous cliffs on the south coast of England with just 20 known sites on the Isle of Portland and one on the Isle of Wight (Blockeel et al. 2014), from where the local vice-county recorder reported *Buddleja davidii* having caused the “virtual loss” of the species. Similarly, the occurrence of (2) *Hydrocharis morsus-ranae* was the only known native site in a north-eastern English vice-county and was considered by the respondent to be “possibly shaded out” by *Lemna minuta* (with low confidence score). Lastly, (3) *Heracleum mantegazzianum* was considered responsible for the extinction of a colony of *Allium oleraceum* in the Scottish Lowlands. A further four species are of ‘Near Threatened’ (NT) status, including reports for *Pilularia globulifera* as threatened by *Crassula helmsii* from three vice-counties. *Pilularia globulifera* has also NT status in the European and global Red Lists of plants (Wyse Jackson et al. 2016). *Cotoneaster integrifolius* agg. was the invasive species most often associated with the direct mentioning of impacts on native species (8 cases), with reports from five vice-counties in southern England, Wales and Ireland, where it was reported to threaten the NT listed species *Gentiana verna* and *Neotinea maculata*. A further European NT species is *Najas flexilis*, for which *Elodea canadensis* was reported to be “Likely responsible for local population extinctions of *Najas flexilis*, *Potamogeton rutilus* (but hard to survey these and be sure)”. Species scoring with higher impacts had generally a

Table 4. Native plant species and their threat category (for Ireland and Great Britain, respectively, depending on location of the vice-county, from Wyse Jackson et al. 2016) stated in the survey to be directly impacted by an invasive plant within the top 10 plants of highest impacts in individual vice-counties, their overall impact, confidence score and region of the vice-county. Threat categories according to IUCN standard: VU = vulnerable, NT = near threatened, LC = least concern.

Impacted species	Threat cat.	Invasive plant	Impact	Confidence	Region
<i>Alchemilla</i> spp.		<i>Alchemilla mollis</i>	Moderate	Low	Northern England
<i>Allium oleraceum</i>	VU	<i>Heracleum mantegazzianum</i>	Major	High	Scottish Lowlands
<i>Allium ursinum</i>	LC	<i>Allium paradoxum</i>	Major	Low	Scottish Lowlands
<i>Allium ursinum</i>	LC	<i>Lysichiton americanus</i>	Massive	Medium	Scottish Lowlands
<i>Butomus umbellatus</i>	LC	<i>Crassula helmsii</i>	Major	High	Southeast England
<i>Cardamine amara</i>	LC	<i>Lysichiton americanus</i>	Massive	Medium	Scottish Lowlands
<i>Carex remota</i>	LC	<i>Allium paradoxum</i>	Major	Low	Scottish Lowlands
<i>Carex remota</i>	LC	<i>Lysichiton americanus</i>	Massive	Medium	Scottish Lowlands
<i>Cerastium</i> spp.		<i>Smyrniolum olusatrum</i>	Massive	High	Southeast England
<i>Chrysosplenium oppositifolium</i>	LC	<i>Allium paradoxum</i>	Major	Low	Scottish Lowlands
<i>Chrysosplenium oppositifolium</i>	LC	<i>Lysichiton americanus</i>	Massive	Medium	Scottish Lowlands
<i>Dryas octopetala</i>	LC	<i>Cotoneaster integrifolius</i> agg.	Major	High	Ireland
<i>Elatine hexandra</i>	LC	<i>Hydrocotyle ranunculoides</i>	Major	High	Southeast England
<i>Fraxinus excelsior</i>	LC	<i>Acer pseudoplatanus</i>	Major	High	Scottish Lowlands
<i>Gagea lutea</i>	LC	<i>Allium paradoxum</i>	Massive	High	Scottish Lowlands
<i>Gentiana verna</i>	NT	<i>Cotoneaster integrifolius</i> agg.	Major	High	Ireland
<i>Gentianella amarella</i>	LC	<i>Cotoneaster integrifolius</i> agg.	Massive	Medium	Southeast England
<i>Helianthemum nummularium</i>	LC	<i>Cotoneaster integrifolius</i> agg.	Massive	Medium	Southeast England
<i>Helianthemum</i> spp.		<i>Cotoneaster integrifolius</i> agg.	Major	Medium	Wales
<i>Helosciadium inundatum</i>	LC	<i>Crassula helmsii</i>	Major	High	Southeast England
<i>Hyacinthoides non-scripta</i>	LC	<i>Hyacinthoides × massartiana</i>	Moderate	Medium	Scottish Lowlands
<i>Hyacinthoides non-scripta</i>	LC	<i>Lamium galeobdolon</i> subsp. <i>argentatum</i>	Massive	High	Scottish Lowlands
<i>Hydrocharis morsus-ranae</i>	VU	<i>Lemna minuta</i>	Major	Low	Northern England
<i>Hypericum elodes</i>	LC	<i>Crassula helmsii</i>	Major	High	Southeast England
<i>Hypericum elodes</i>	LC	<i>Hydrocotyle ranunculoides</i>	Major	High	Southeast England
<i>Lamium galeobdolon</i> subsp. <i>montanum</i>	LC	<i>Lamium galeobdolon</i> subsp. <i>argentatum</i>	Major	High	Midlands
<i>Lysimachia nemorum</i>	LC	<i>Allium paradoxum</i>	Major	Low	Scottish Lowlands
<i>Medicago</i> spp.		<i>Smyrniolum olusatrum</i>	Massive	High	Southeast England
<i>Najas flexilis</i>	LC	<i>Elodea canadensis</i>	Major	High	Scottish Highlands & Islands
<i>Neotinea maculata</i>	NT	<i>Cotoneaster integrifolius</i> agg.	Massive	High	Ireland
<i>Neotinea maculata</i>	NT	<i>Cotoneaster integrifolius</i> agg.	Major	High	Ireland
<i>Pilularia globulifera</i>	NT	<i>Crassula helmsii</i>	Moderate	Low	Wales
<i>Pilularia globulifera</i>	NT	<i>Crassula helmsii</i>	Major	Medium	Wales
<i>Pilularia globulifera</i>	NT	<i>Crassula helmsii</i>	Major	Medium	Midlands
<i>Potamogeton rutilus</i>	LC	<i>Elodea canadensis</i>	Major	High	Scottish Highlands & Islands
<i>Potamogeton praelongus</i>	NT	<i>Elodea canadensis</i>	Major	High	Scottish Highlands & Islands
<i>Ranunculus baudotii</i>	LC	<i>Azolla filiculoides</i>	Massive	High	Southwest England
rare calcicoles		<i>Quercus cerris</i>	Major	Medium	Wales

Impacted species	Threat cat.	Invasive plant	Impact	Confidence	Region
<i>Rubus dasycarpus</i>		<i>Rubus armeniacus</i>	Massive	High	Northern England
<i>Salicornia</i> spp.		<i>Spartina anglica</i>	Massive	High	Southeast England
<i>Salix</i> spp.		<i>Rubus spectabilis</i>	Major	High	Scottish Highlands & Islands
<i>Sambucus nigra</i>	LC	<i>Sambucus racemosa</i>	Minor	Medium	Scottish Lowlands
<i>Micranthes nivalis</i>	LC	<i>Epilobium brunnescens</i>	Moderate	High	Scottish Highlands & Islands
<i>Sedum anglicum</i>	LC	<i>Sedum album</i>	Moderate	High	Northern England
<i>Southbya nigrella</i>	VU	<i>Buddleja davidii</i>	Major	High	Southeast England
<i>Thymus</i> spp.		<i>Cotoneaster integrifolius</i> agg.	Massive	Medium	Southeast England
<i>Trifolium</i> spp.		<i>Smyrniolum olusatrum</i>	Massive	High	Southeast England
<i>Ulmus glabra</i>	LC	<i>Acer pseudoplatanus</i>	Major	High	Scottish Lowlands

higher distribution in the vice-county where they were scored than species with lower distributions (Fig. 4), (ANOVA, $F(4) = 7.90$, $p < 0.001$). Furthermore, across all vice-counties, species that were included in a county’s top 10 list were recorded from an average of 15.5% of the county’s total number of grid cells, whereas in vice-counties where the species were not included in the top list, they were recorded on the average of 10.1% grid cells (two sample t-test, $t = -7.96$, $p < 0.001$).

Discussion

Our results demonstrate the value of local experts’ contributions to impact assessments of non-native species. This is the first study bringing together assessments by local volunteer experts, based on their own field experience with a national assessment conducted by researchers relying mainly on published evidence. The analysis confirms the results of the national assessment, but also adds a new level of evidence which will be relevant for future national assessments, as well as local and regional management decisions. Here, we discuss how our approach could complement and feed into comprehensive impact assessments, how the results compare to previous assessments in Britain and Ireland and how our local experts’ contributions go beyond the identification of species to include information on impacts on habitats and native species.

Integration of local knowledge into larger scale impact assessments and working with EICAT

The aim of our study was not to conduct an impact assessment following the full EICAT framework process. Our study was based on local volunteer experts reporting non-native species relying on their observations of the impacts of species in areas they are familiar with. In contrast, the EICAT approach is based on experts assessing published evidence on species’ impacts including impact mechanisms (Hawkins et al. 2015). Our respondents’ scoring of impacts was based on their observations and

individual perceptions and lacks the rigour underlying published evidence. However, both approaches connect through using the EICAT assessment categories and criteria. The value of our approach lies in that it taps into a pool of knowledge that would often not make it into the published evidence assessed in the EICAT process. Species with impacts may thus be recognised earlier, because it is usually local experts that will notice changes in their environment first. Many of these records may take a long time from there to a professional investigating and publishing these impacts or may not be reported at all. This is illustrated by the number of species reported in our study that have not been considered in the GB national assessment. Studies such as ours could, therefore, be useful to feed into full EICAT assessments at national levels, not just for plants, but also for other species groups where local experts are involved in recording and monitoring. Future studies could also benefit by including a question on impact mechanisms as in the EICAT assessment, although there is always a risk that, if responding to a survey takes too much time, the response rate is likely to decrease.

Our results showed that the EICAT impact categories could be well communicated to local experts and were well-suited to be used by them. We, therefore, argue that our results are, to some extent, comparable to impact assessments using the same categories and criteria and we have confirmed this by comparing our results to the GB national level expert assessment. Ranking of species in the order they were submitted (highest impact species first) produced very similar results to the scoring using the assessment criteria. We would, nevertheless, not conclude that similar studies should just ask for a ranked list of species without providing impact definitions, as these will have guided respondents in their choice of species. However, the ranking is useful considering different interpretation of the criteria in the assessment scheme, as well as in the perceptions of impacts by individual respondents.

Comparison to the national level GB scoring and previous assessments for Ireland

We achieved a remarkably similar list to the species list in the expert assessment at GB national level. Species absences in our top list are explained by the fact that coastal habitats are not present in many areas from which we received responses (in the case of *Spartina anglica*) or because they were outside the taxonomic scope of vice-county recorders (e.g. *Campylopus introflexus* was outside the scope of most recorders). The study also identified species that were not included in the top list of species in the national impact scoring scheme, for example, *Picea sitchensis*, *Lamiastrum galeobdolon* subsp. *argentatum* and *Prunus laurocerasus* all ranked highly in our study, but did not achieve higher scores in the GB scoring, with *P. laurocerasus* ranked ‘moderate’ and the other two species ‘minor’. However, the GB national assessment also asked reviewers to score the potential maximum impact (an option which was not offered in our project), defined as the impact a species “would be expected to have in GB if it were established in all parts that are suitable (i.e. based on current biotic and abiotic conditions)” (Booy 2019) and all three

species were scored ‘major’. This result could indicate that the evidence used in the GB scoring was out-of-date and the species’ more extensive impacts have not been documented so far or accessible to the national evaluators, who, nevertheless, realised the potential for higher impacts of these species in their potential maximum impact score.

For Ireland, the survey reported 12 species on the list of 16 high impact species identified by Kelly et al. (2013), based on risk assessments and nine of the species listed by Gioria et al. (2018), based on a literature review of terrestrial plants. Species included in these two sources, but not reported in our survey for Ireland, include *Reynoutria sachalinensis*, *Hydrocotyle ranunculoides*, *Carpobrotus edulis* and *Crassula helmsii*. Species reported in our survey with ‘massive’ impacts, but missing in Kelly et al. (2013) are *Picea sitchensis*, *Rubus spectabilis* and *Cotoneaster integrifolius*, with the latter two being also identified by Gioria et al. (2018). Furthermore, two species included in the top ten list of species reported from Ireland in our survey, *Allium triquetrum* and *Pinus contorta*, are not included in either of these two references.

Scoring consistency and suitability of the approach for future assessments

We found scoring consistency differed for individual species between vice-county recorders. This can be attributed to differences inherent to individual respondents and how they interpreted and applied the assessment criteria. Vice-county recorder may be in their roles for various lengths of time and may have different levels of commitment and experience, which could influence their scoring. Similar, their perception of the impacts of species in their vice-counties and of invasive species, in general, could influence how they scored, with underlying factors not studied in our analysis informing individual people’s perceptions (Shackleton et al. 2019). However, it is also likely that these differences can, to some extent, be explained by different introduction and spread histories of the species and/or the availability of habitats in different vice-counties. González-Moreno et al. (2019) investigated patterns of consistencies in scoring between different assessors for several invasive species and impact assessment schemes including EICAT and found, on average, a coefficient of variation (CV) of 40%. In comparison, the 32% variation for the impact score of invasive plants in our study is lower, which could be the result of higher numbers of scores for most species (i.e. between 4 to 63, compared to 5 to 8 in González-Moreno et al. 2019), but it is also reassuring to see a comparable level of agreement at the species level, despite the fact that respondents reported from different local areas. However, we also found clear differences in scoring between different respondents, ranging from one respondent reporting 10 species all with ‘massive’ impacts for their vice-county to another one who had scored just one species (*Allium paradoxum*) as ‘moderate’, with the remaining nine all as either ‘minor’ (3 species) or ‘minimal’ (6). Overall, variation between recorders was 25% (CV).

Habitats and species affected

While most records of non-native plants in Europe and the British Isles are from human-made habitats (i.e. industrial habitats and arable land, parks and gardens) (Lambdon et al. 2008; Stace and Crawley 2015), impacts of species in our survey were most

frequently associated with woodlands. This seems reasonable, given that we asked respondents to consider environmental impacts only, which will have excluded many impacts of invasive species in human-made habitats. Linear features and boundaries were the second most mentioned habitat type; however, this habitat is more difficult to interpret as it encompasses a wide range of different linearly-arranged landscapes elements according to the UK broad habitat classification, ranging from hedgerows, tree lines and walls to dry ditches (Jackson 2000). Nevertheless, linear features are recognised to play an important role in supporting biodiversity and ecosystem services (Holland et al. 2016; Phillips et al. 2020) and the high number of impacting non-native species are a reason for concern. Equally concerning are the high number of species reported from freshwater and wetland habitats combined with the fact that 11 of the 14 aquatic species in the non-native flora of the British Isles have been reported. Urban and brownfield sites, many of which are also well known to support biodiversity (Harrison and Davies 2002; Venn et al. 2013), feature less species, but this could also be the result of these habitat types not specifically mentioned in the UK broad habitat type categorisation and, therefore, not included in our survey design.

Conclusions

Our study provides evidence that local experts can make highly valuable contributions to the assessment of impacts associated with invasive non-native species. This contribution lies not only in the confirmation of results from national assessments, but also in identifying less well documented, but widely distributed species, as well as species with limited occurrences and high impacts locally.

Our assessment gained in value by adding a question in our survey about the main habitat type and native species affected. This information will further help to understand invasive species' impacts and guide management, in particular, at more local levels. Furthermore, the results provide a valuable baseline which can be followed up by surveys in regular intervals (for example 5–10 years) to document changes in the spectrum of species, as well as the reported impacts and habitats and native species affected. For this purpose, it is also important to have used the internationally recognised EICAT impact categories as a standard which will help to maintain a good level of consistency at the temporal scale in the future and integration into national impact assessments.

Our results will also be useful to provide information for national and local planning and policies, risk assessments and management actions, as well as directing future research.

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Supplementary material I

Survey and Tables S1–S3

Authors: Katharina Dehnen-Schmutz, Oliver L. Pescott, Olaf Booy, Kevin J. Walker

Data type: Questionnaire form.

Explanation note: **Part 1:** Questionnaire survey text **Part 2:** Table S1 (Diversity at regional levels), Table S2 (results of the post-hoc row wise Fisher test comparing the proportions of lifeform), Table S3 (number of observations of invasive plants affecting different habitats as reported in the survey).

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Link: <https://doi.org/10.3897/neobiota.77.89448.suppl1>

Supplementary material 2

Table S2

Authors: Katharina Dehnen-Schmutz, Oliver L. Pescott, Olaf Booy, Kevin J. Walker

Data type: Csv file.

Explanation note: Survey results and species information.

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Link: <https://doi.org/10.3897/neobiota.77.89448.suppl2>

Supplementary material 3

Figure S1

Authors: Katharina Dehnen-Schmutz, Oliver L. Pescott, Olaf Booy, Kevin J. Walker

Data type: Image.

Explanation note: **Figure S1.** Bipartite graph illustrating which species have been reported to have impacts in which habitats weighted by the sum of the impacts of each species reported for the respective habitat. Littoral habitat categories have been combined.

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Link: <https://doi.org/10.3897/neobiota.77.89448.suppl3>

Variation in plant traits and phylogenetic structure associated with native and nonnative species in an industrialized flora

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Abstract

Industrialized sites are hotspots for nonnative species because of continuous anthropogenic disturbance and nonnative propagule rain resulting from hitchhikers exchanged through global trade. Investigating plant traits and the phylogenetic structure of species at initial ports of entry can contribute to our understanding of how species are introduced to, assembled into, and survive at industrialized sites, which can also inform how susceptible these sites are to nonnative plant invasions. To compare native and nonnative species, we asked three questions: 1) Are plant traits differentially associated with species nativity (native versus nonnative)? (2) Do these traits have phylogenetic signals? and (3) What is the phylogenetic structure of each trait for native and nonnative species? We collected, identified, and vouchered 170 angiosperm species within the Garden City Terminal at the Port of Savannah, Georgia, USA, the largest container terminal in North America. Species nativity was derived from the literature, as were traits of pollination syndrome, dispersal syndrome, duration, and growth habit. Pearson's Chi-squared tests were used to determine if traits were differentially associated with species nativity. Phylogenetic signal, along with mean pairwise distance (MPD) and mean nearest taxon distance (MNTD), were used to assess the degree of phylogenetic relatedness of native and nonnative species with each trait. Nonnative species showed a significant association with multiple pollination syndromes. Native species were significantly associated with perennial duration and zoophily pollination syndrome. All traits possessed a phylogenetic

signal, and the anemophily pollination syndrome was significantly clustered for both native and nonnative species. Still, most other traits differed in their phylogenetic structure pattern based on the nativity. Overall, findings suggest that the environment is filtering for native and nonnative species that possess traits promoting introduction and survival at this industrialized point-of-entry. They also suggest that nonnative species trait differences partition available niches that promote their introduction to the site. More research is needed at industrialized sites to inventory and monitor the floristic community, investigate the establishment and spread probabilities of nonnative species, and prevent and mitigate nonnative species risks and impacts.

Keywords

global trade, industrialized flora, phylogenetic structure, species nativity, trait associations

Introduction

Approximately 13% (47,840) of all vascular plant species have the potential to become nonnative species in new environments (Seebens et al. 2018). As the world becomes increasingly connected and the introduction of nonnative species accelerates, there is an increasing need to prevent and mitigate risks of potentially successful species invasions through early-detection and rapid-response (**EDRR**) efforts (Bezeng et al. 2013; Seebens et al. 2017; Pyšek et al. 2020; Seebens et al. 2021). Upon arrival at a novel range, nonnative species may establish after the transport and introduction phases of the biological invasion framework (Blackburn et al. 2011; Harris et al. 2018). These new arrivals, commonly pre-adapted to disturbance and ruderal in nature (Davis et al. 2000), can then spread in a new terrestrial landscape of varied niches formed by human activities (Cadotte and Lovett-Doust 2001; Dolan et al. 2017; Borden and Flory 2021). Investigating plant traits of currently present nonnative species and mapping those traits onto a phylogeny to compare with established native species are key to improving our understanding of how the environment and human-disturbance in new landscapes can select for certain traits. Results from studies comparing traits and phylogenies between native and nonnative species can then be used to inform assessments of industrialized flora (Lucardi et al. 2020a) and to predict nonnative species' capacity to become invasive at initial introduction sites (Lucardi et al. 2020b).

Industrialized sites experience extraordinarily high levels of human activity and disturbance, creating environmental constraints that limit plant occupancy (Williams et al. 2009; Kowarik and von der Lippe 2018; Knapp et al. 2022). Heavily and continuously disturbed industrialized sites (immature novel ecosystems, as defined by Kowarik and von der Lippe 2018) are generally open-canopy locales with intense solar radiation, pollutants, and limited substrate availability. Often, these human-dominated sites are linked to the introduction of nonnative species (Hobbs and Huenneke 1992; Crawley et al. 1996; Cadotte and Lovett-Doust 2001; Daehler 2003; Tsang et al. 2019; Lucardi et al. 2020b). However, both native and nonnative species arriving in industrialized environments may require specific traits conducive to their survival and

potentially longer-term establishment that for nonnative species may result in invasive spread (Funk and Vitousek 2007; Cadotte et al. 2017; Borden and Flory 2021).

In an industrialized flora (Lucardi et al. 2020a), traits deemed important for the successful introduction and survival of native and nonnative species include dispersal and pollination syndromes, growth habit, and duration (lifespan) (Pyšek et al. 1995; Pyšek et al. 2008; Borden and Flory 2021). Certain strategies involving these traits have been previously linked to the ability of plant species to occupy and survive in urban areas and industrialized sites (Williams et al. 2015; Palma et al. 2017; Knapp et al. 2022). For example, species with wind dispersal and wind pollination (Williams et al. 2015; but see Palma et al. 2017) have been found at higher frequencies in urban habitats than species with other dispersal mechanisms; this may be due to increased air turbulence (Knapp et al. 2010) and the suitability of these habitats to other dispersal and pollination mechanisms (Lososová et al. 2006). Frequently, disturbed sites are associated with annual species (Palma et al. 2017), whose lifespans are less affected by disturbances than biennial and perennial species (Knapp et al. 2022). Interestingly, in studies that have compared the traits of native and nonnative species, some have found similar or shared traits (Leishman et al. 2010; Tecco et al. 2010; Lemoine et al. 2015), whereas others have found dissimilar traits linked to species nativity (Pyšek and Richardson 2008; van Kleunen et al. 2010; Godoy et al. 2011).

Uncertainty surrounding which traits are linked to species' success in new environs limits understanding of how invasion happens and how to predict it (Ehrenfeld 2010; Drenovsky et al. 2012; Catford et al. 2019; Palma et al. 2021). Previous studies have highlighted wind pollination (Andersen 1995), fleshy fruits (Andersen 1995), and taller plant height (Crawley et al. 1996; Williamson and Fitter 1996; Montesinos 2021) as important plant traits for invasion success, but other studies have found contrasting results. For example, some research has noted a stronger link between nonnative species and animal or self-pollination than wind pollination (Williamson and Fitter 1996; Cadotte and Lovett-Doust 2001; Milanović et al. 2020). Investigation of key traits that promote the introduction and survival of nonnative species at initial arrival sites could illuminate the formation of species composition at industrialized or urban sites. Nonnative species traits should also be compared to the traits of native species to address the similarities or differences among those in high-disturbance, industrialized environments (Funk and Vitousek 2007; Loiola et al. 2018).

Analyses of phylogenetic signal and structure can be used to understand which traits facilitate the assembly of native and nonnative species in industrialized communities. For instance, the presence of phylogenetic signals would indicate the degree to which phylogenetic similarity predicts trait similarity in the community (Yang et al. 2014). Phylogenetic structure analyses can highlight the phylogenetic distribution of native and nonnative species with certain traits in the community (Loiola et al. 2018). For example, the finding of an over-dispersed pattern for a trait may highlight niche partitioning of species along the trait axis (Funk et al. 2008; Liu et al. 2013; Lemoine et al. 2015). A clustered pattern for a trait may indicate habitat filtering that favors certain traits over others (Funk et al. 2008; Liu et al. 2013; Lemoine et al. 2015) or

performance differences between species (Mayfield and Levine 2010). These phylogenetic patterns can highlight if traits of native and nonnative species have similar or dissimilar phylogenetic distributions, plant traits or lineages favored at industrialized sites, and the potential processes that lead to their assembly at these sites (Dolan et al. 2017).

Industrialized sites, such as the Garden City Terminal, the primary container handling facility of the Port of Savannah, present an ideal laboratory to investigate traits and phylogenetic distributions of native and nonnative species under continuous, active disturbance. At the Garden City Terminal's green spaces, Lucardi et al. (2020a) presented the stark contrast between plant species richness of native and nonnative species. They found that nonnative species richness and proportional nonnative to native ratios at this industrialized site were higher when compared to other floras in the same region. Herein, we addressed the following questions from the vascular plants collected in Lucardi et al. (2020a): (1) Are traits differentially associated with species nativity? (2) Do these traits have phylogenetic signals? and (3) What is the phylogenetic structure of each trait for native and nonnative species? We predict that traits vary in their association with native and nonnative species, traits have phylogenetic signals, and patterns of phylogenetic structure of species' traits differ between native and nonnative species. Based on these questions, we hope to determine how trait differences contribute to the introduction of nonnative species at this industrialized site.

Methods

Study site

This study was conducted at the Port of Savannah, Georgia, USA (32°07.3'N, 81°08.4'W). At the port, we specifically focused on the Garden City Terminal (**GCT**), the main container-handling terminal that spans 485.6 hectares and is primarily composed of impervious surfaces (i.e., asphalt) interspersed with small green spaces for water run-off (Lucardi et al. 2020a). The Port of Savannah has a subtropical climate and is in the USDA plant hardiness zone 8b (USDA Plant Hardiness Zone Map 2012). This hardiness zone is characterized by average annual minimum temperatures ranging from -9.4 °C to -6.7 °C (Daly et al. 2012). During the sampling period (2015–2017), the average temperature was 21.1 °C (NOAA 2021).

Floristic and trait inventory

The flora was sampled from six green spaces (4.51 ha, ~1% of the GCT) on four separate occasions between August 2015 and February 2017 to capture seasonal changes in the flora (see Lucardi et al. 2020a for details). All accessible angiosperm species in flower or fruit were collected during each sampling date. Vouchers from these repeated surveys were identified, and dried specimens were deposited into the Arkansas State University Herbarium (**STAR**) and Columbus State University Herbarium (**COLS**). Our collection comprises 174 species, representing 130 genera and 51 families. To

make all investigated plant traits and phylogenetic structure patterns comparable, we only consider angiosperms (170 species), removing two fern species, *Asplenium platyneuron* (L.) Britton, Sterns & Poggenb and *Lygodium japonicum* (Thunb.) Sw., a notable nonnative and pyrogenic invasive plant, along with two gymnosperm species (*Juniperus virginiana* L. and *Pinus taeda* L.).

Information on traits was gathered from the literature (Fig. 1; Suppl. material 1). Nativity (Native/Nonnative) was previously reported in Lucardi et al. (2020a) for this flora. The other four traits included 1) dispersal syndrome, 2) pollination syndrome, 3) duration, and 4) growth habit. Most of this information was gathered from the USDA PLANTS Database (USDA NRCS 2021). Missing information was supplemented by the literature for any particular species (Suppl. material 1). All traits are categorical. Dispersal syndrome was composed of five categories, according to Schulze et al. (2019): anemochory (wind dispersal), autochory (self-dispersal), hydrochory (water dispersal), zoochory (animal dispersal), and polychory (multiple dispersal mechanisms). Pollination syndrome was also composed of five categories, according to Yang et al. (2020): anemophily (wind pollination), hydrophily (water pollination), self-pollination, zoophily (animal pollination), and multiple (multiple pollination mechanisms). There were four duration categories: annual, biennial, perennial, and multiple (multiple duration strategies). Lastly, there were six categories of growth habit: forb, graminoid, subshrub, tree, vine, and multiple (multiple growth strategies). Duration and growth habit categories were defined by those on the USDA PLANTS Database (USDA NRCS 2021).

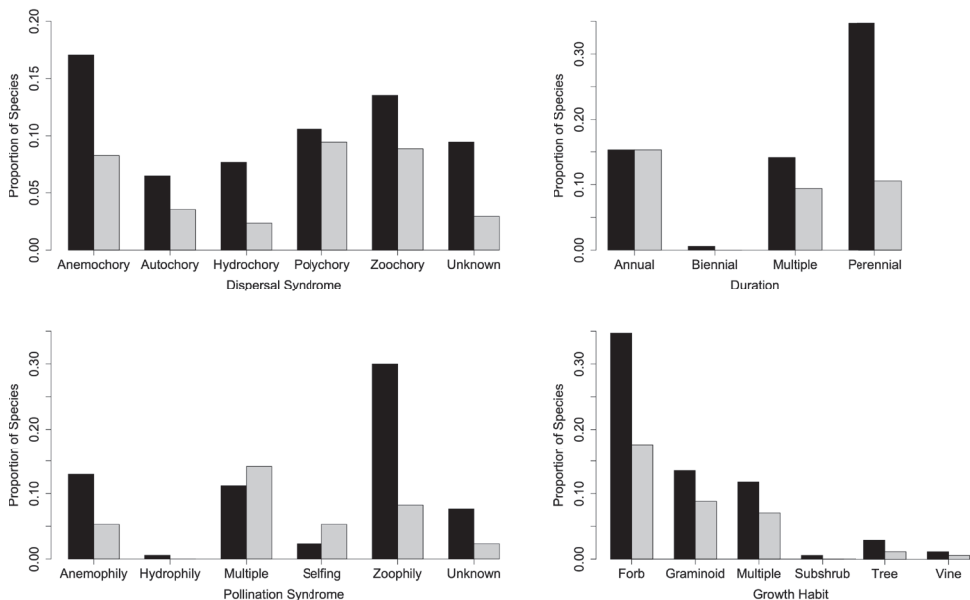


Figure 1. The proportion of all species in the dataset in each category of each trait investigated. The flora consists of 170 species comprised of 110 native and 60 nonnative species. Black bars represent the proportion of native species and gray bars represent the proportion of nonnative species in all figure panels.

Trait data analysis

Pearson's Chi-squared tests were used to determine if traits were differentially associated with species nativity, with the null hypothesis assuming independence of traits and nativity. For analyses exhibiting significant differences, we performed *post hoc* analyses considering all combinations of trait categories and species nativity using the `chisq.posthoc.test` package (Ebbert 2019) in R statistical software (R Core Team 2021). Categories with low sample sizes were eliminated from analyses, including removing biennial (1) from the duration trait, hydrophily (1) from the pollination syndrome trait, and subshrub (1), vine (3), and tree (7) categories from the growth habit trait.

DNA barcode analysis: DNA isolation, PCR amplification, and sequencing

DNA barcoding of the flora was performed to build a phylogenetic tree for analyses and for confirmation of species identifications (Whitehurst et al. 2020). Leaf samples were taken from each plant specimen and stored on silica gel until processing. All DNA barcode analyses were conducted at the Canadian Centre for DNA Barcoding (CCDB), Biodiversity Institute of Ontario, Canada, including DNA extraction (Ivanova et al. 2008; Ivanova et al. 2016), PCR amplification (Kuzmina and Ivanova 2011; Fazekas et al. 2012), and sequencing (Ivanova and Grainger 2006; Kuzmina and Ivanova 2006). Ribulose-bisphosphate/carboxylase large subunit (*rbcL*) and maturase-K (*matK*) gene regions of the chloroplast genome were sequenced using forward and reverse primers and Sanger sequencing technology (Levin et al. 2002; Kress et al. 2009). In instances where sequencing failed, publicly available sequences were used, when available, from the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007) or GenBank (Benson et al. 2013). All sequences generated in this study are publicly available on BOLD and GenBank (Suppl. material 2). Only species with both DNA barcode gene regions were included in phylogenetic analyses. We eliminated nine species that either (1) amplified only the *rbcL* or *matK* region or (2) failed to sequence completely (Suppl. material 2), resulting in 161 plant species for inclusion in phylogenetic analyses. Sequences for an additional two species placed them into questionable areas of the phylogenetic tree, causing us to question their tissue sample used in sequencing, so we eliminated them from phylogenetic analyses. The final dataset for phylogenetic analyses consisted of 159 species (Suppl. material 3).

Phylogenetic tree reconstruction

Sequences of the *rbcL* and *matK* gene regions were aligned separately using multiple alignment and fast Fourier transform (MAFFT v 7.471) with the FFT-NS-2 algorithm (Katoh and Standley 2013). The alignments were then concatenated into a super-

matrix from which a phylogeny was generated. A phylogeny was constructed using maximum likelihood (ML) methods in the “phangorn” package (Schliep 2011) in R statistical software (R Core Team 2021) with *Ginkgo biloba* L. as the outgroup. Nucleotide substitution was modeled using the general time-reversible substitution model with gamma-distributed rate variation and a proportion of invariant sites (GTR + Γ + I). Node support was estimated from 1000 bootstrap replications.

Phylogenetic signals and structure

We quantified phylogenetic signals in the four traits to determine the degree to which the phylogenetic tree estimates the similarity of species traits. Phylogenetic signals were determined by quantifying the parsimony Sankoff score calculated from the distribution of trait categories on the phylogeny (Maddison and Slatkin 1991) using the “phangorn” package (Schliep 2011) in R statistical software (R Core Team 2021). The significance of the Sankoff score was determined by randomly shuffling the species on the tips of the phylogeny 999 times to generate a null distribution that was compared to the observed parsimony score to calculate a P-value. A P-value < 0.05 was indicative of closely related species having similar traits.

We also calculated the mean pairwise distance (**MPD**) and mean nearest taxon distance (**MNTD**) using the “picante” package (Kembel et al. 2010) in R statistical software (R Core Team 2021). These metrics measure the degree of phylogenetic relatedness of species in defined groups. In this study, groups were made comparing native and nonnative species groups for each trait category. For example, for the dispersal syndrome trait, species were grouped into native or nonnative for each of the categories (anemochory, autochory, hydrochory, zoochory, and polychory). In this instance, MPD is the mean phylogenetic distance among all pairs of species within a group and captures the overall phylogenetic dissimilarity of the taxa in the group of interest (Swenson 2014). The mean distance between each species within a group and its closest relative is expressed as MNTD. The two metrics provide complementary information for which MPD is focused on the whole group phylogeny, and MNTD captures finer scale phylogenetic patterns, making it more sensitive to sister-taxon distances and the length of the tips of the phylogeny (Swenson 2014; Cadotte et al. 2018; Loiola et al. 2018).

For MPD and MNTD, observed values were compared to null distributions generated by randomizing the names of the taxa on the phylogenetic distance matrices 999 times to calculate standardized effect sizes (SES) and P-values (quantiles). Negative SES values (obs.z < 0) and low quantiles (obs.p < 0.05) for both MPD and MNTD indicated species in a group are phylogenetically clustered, with smaller phylogenetic distances among the species in the group than expected (Swenson, 2014). Positive SES values (obs.z > 0) and high quantiles (obs.p > 0.95) indicated species in a group are phylogenetically over-dispersed, with greater phylogenetic distances among species in the group than expected (Swenson 2014).

Results

Relationships between traits and species nativity

Of the 170 species in this study flora, 110 were native to the southeastern region of the USA, and 60 were nonnative (Appendices S1 and S3). Significant differences in association were found between two of the four traits and species nativity (Fig. 1, Table 1). Pollination syndrome resulted in a significant association with species nativity ($\chi^2 = 19.867$, $df = 3$, $P < 0.01$). *Post hoc* analysis revealed significant associations between the categories multiple ($P = 0.02$) and zoophily ($P = 0.01$), and species nativity (Table 1). The multiple pollination strategy was strongly positively associated with nonnative species, and zoophily was strongly positively associated with native species. Duration was also significantly associated with species nativity ($\chi^2 = 10.07$, $df = 2$, $P < 0.01$). The perennial ($P = 0.02$) duration category was significantly associated with species nativity according to *post hoc* analysis (Table 1). Nonnative species had a strong positive association with annual duration and a strong negative association with perennial duration. Native species showed opposite and weaker association patterns with annual and perennial duration categories compared to nonnative species. The overall χ^2 score was largely influenced by annual (31%) and perennial (32%) nonnative species. These categories contributed most to the overall χ^2 score because they had far fewer observed species than expected.

Dispersal syndrome was not significantly associated with species nativity ($\chi^2 = 3.29$, $df = 4$, $P = 0.51$). However, there were still differences in the signs of association between trait categories and species nativity. Native species had positive associations with anemochory and hydrochory dispersal syndromes but negative associations with

Table 1. Representation of association found between traits and species nativity (native and nonnative). Significant associations (bolded) were determined from Pearson’s Chi-squared tests with *post hoc* analyses. The sign of association between each trait category and nativity are given, negative (–) and positive (+). Trait categories with significant phylogenetic structure are also displayed. Trait categories with significant phylogenetic clustering are denoted by ^. Trait categories with significant phylogenetic over-dispersion are denoted by *.

Trait	Trait Category	Native	Nonnative
Dispersal Syndrome	Anemochory	+ ^	–
	Autochory	+	– ^
	Polychory	– *	+ *
Pollination Syndrome	Anemophily	+ ^	– ^
	Multiple	–	+
	Selfing	–	+ *
	Zoophily	+ ^	–
Duration	Multiple	– ^	+
	Perennial	+ ^	–
Growth Habit	Forb	+ ^	– ^
	Graminoid	– ^	+ ^
	Multiple	– ^	+

polychory and zoochory dispersal syndromes. Nonnative species had opposite associations with the dispersal syndrome categories as native species. Overall, polychory (native: 17%; nonnative: 29%) contributed the most to the total χ^2 score. There was also no significant association between growth habit and species nativity ($\chi^2 = 0.59$, $df = 3$, $P = 0.90$). The graminoid category contributed the most (native = 18%, non-native = 32%) to the overall χ^2 score.

Phylogenetic signals and structure

We quantified the observed parsimony Sankoff score for each trait and compared it to a null distribution of parsimony scores to determine significance. We found significant phylogenetic signals for all four traits (pollination and dispersal syndromes, growth habit, and duration), with close relatives generally sharing more similar traits than expected by chance (Table 2). Phylogenetic structure analyses investigated the structure of native and nonnative species within each category of each trait (Table 3). For native species, anemochory and polychory dispersal syndromes showed significant phylogenetic structure. The standardized effect sizes of MPD and MNTD showed evidence of phylogenetic clustering for anemochory, whereas MNTD showed evidence of over-dispersion for polychory (Tables 1, 3). Standardized effect sizes of MNTD also showed evidence of over-dispersion for polychory for nonnative species (Tables 1, 3). Autochory dispersal syndrome was found to be significantly, phylogenetically clustered for nonnative species according to MPD and MNTD (Tables 1, 3). Standardized effect sizes of MPD and MNTD supported significant phylogenetic clustering for zoophily, while MNTD supported significant phylogenetic clustering for the anemophily categories of pollination syndrome in native species (Tables 1, 3). For nonnative species, standardized effect sizes of MPD and MNTD showed significant phylogenetic clustering for anemophily, where MNTD supported significant phylogenetic over-dispersion for the selfing category of pollination syndrome (Tables 1, 3). Only native species showed any significant phylogenetic structure for the duration (lifespan) trait, with MPD and MNTD showing evidence of phylogenetic clustering for multiple and perennial duration categories, respectively (Tables 1, 3). Finally, for the growth habit trait for native species, significant phylogenetic clustering was supported by MPD and MNTD for the forb and graminoid categories, while MPD provided support for the multiple growth habit category (Tables 1, 3). Significant phylogenetic clustering was also supported for the forb (MPD) and graminoid (MPD and MNTD) categories based on standardized effect sizes for nonnative species (Tables 1, 3).

Table 2. Phylogenetic signals using parsimony Sankoff scores. All P-values were significant (< 0.05).

Traits	Sankoff	n	P
Dispersal Syndrome	84	139	<0.01
Pollination Syndrome	69	142	<0.01
Duration	81	159	<0.01
Growth Habit	60	158	<0.01

Table 3. Phylogenetic structure within each of the categories of each of the four traits. MPD.obs.z is the standardized effect size of the mean pairwise distance measurement. MNTD.obs.z is the standardized effect size of the mean nearest taxon distance measurement. Standardized effect sizes were calculated from comparisons of observed values to null distributions generated by randomizing the names of the taxa in the phylogenetic distance matrices 999 times. P-values in bold are significant. Significant phylogenetic clustering is denoted by ^ ($P < 0.05$). Significant phylogenetic over-dispersion is denoted by * ($P > 0.95$).

Trait	ntaxa	MPD.obs.z	P	MNTD.obs.z	P
Dispersal Syndrome					
Anemochory	24	-7.19	0.001 [^]	-2.40	0.012 [^]
Anemochory.NN	14	0.27	0.572	-0.92	0.178
Autochory	11	-0.73	0.226	0.46	0.679
Autochory.NN	6	-1.95	0.049 [^]	-1.88	0.034 [^]
Hydrochory	12	0.98	0.855	-0.84	0.199
Hydrochory.NN	4	0.52	0.660	-0.79	0.767
Polychory	18	0.93	0.812	1.99	0.972 [*]
Polychory.NN	15	2.23	0.997 [*]	-0.38	0.365
Zoochory	22	-1.47	0.080	-1.24	0.110
Zoochory.NN	13	0.80	0.788	-0.99	0.169
Pollination Syndrome					
Anemophily	18	0.51	0.683	-2.72	0.004 [^]
Anemophily.NN	9	-4.25	0.001 [^]	-2.19	0.016 [^]
Multiple	18	-0.41	0.322	0.66	0.746
Multiple.NN	24	0.22	0.542	-1.14	0.130
Selfing	4	0.70	0.721	1.23	0.883
Selfing.NN	9	2.04	0.994 [*]	0.49	0.676
Zoophily	47	-5.47	0.001 [^]	-1.98	0.024 [^]
Zoophily.NN	13	-1.71	0.057	0.43	0.657
Duration					
Annual	27	-1.23	0.124	-1.57	0.060
Annual.NN	25	1.18	0.885	-1.27	0.115
Multiple	22	-3.72	0.002 [^]	0.20	0.576
Multiple.NN	16	1.30	0.927	-0.41	0.334
Perennial	52	-0.23	0.383	-1.95	0.025 [^]
Perennial.NN	17	-0.201	0.377	0.16	0.556
Growth Habit					
Forb	56	-7.34	0.001 [^]	-2.34	0.009 [^]
Forb.NN	29	-2.00	0.035 [^]	-1.15	0.131
Graminoid	18	-4.69	0.001 [^]	-4.75	0.001 [^]
Graminoid.NN	15	-6.87	0.001 [^]	-3.85	0.001 [^]
Multiple	20	-2.95	0.007 [^]	-1.23	0.112
Multiple.NN	10	-0.64	0.253	-0.12	0.446
Tree	5	-1.77	0.052	-0.37	0.360
Tree.NN	2	-0.57	0.268	-0.64	0.246
Vine	2	-1.27	0.101	-1.23	0.106
Vine.NN	1	NA	NA	NA	NA

Discussion

This research is part of an ongoing, innovative research initiative to quantify and assess plant communities within industrialized initial points-of-entry sites (Lucardi et al. 2020a), and to directly measure the diversity and phenology of propagule pressure and model risk of establishment (Lucardi et al. 2020b). Here, we investigated 1) the traits

of native and nonnative plant species from the industrialized flora located at the GCT of the Port of Savannah, Georgia, USA, 2) quantified associations between species traits and their nativity (native or nonnative), and 3) evaluated phylogenetic structure patterns of these traits. Consistent with our first prediction, we found that native and nonnative species were associated with different traits. Importantly, nativity was significantly associated with different categories of pollination syndrome and duration. We also found that all traits had phylogenetic signals, supporting our second prediction. Lastly, we found mixed support for our third prediction, as all but two trait categories with significant phylogenetic structure differed between native and nonnative species.

Native species in the flora at the Garden City Terminal of the Port of Savannah showed significant, positive associations with perennial duration and zoophily pollination syndrome (Table 1). Previous research into these species at this site also noted their adaptation to weedy and disturbed habitats (based on Coefficients of Conservatism [CofC], Lucardi et al. 2020a). Research into the southwestern Ontario flora, with 27% nonnative species, previously found anemochory, zoophily, and perennial duration to be associated with native species (Cadotte and Lovett-Doust 2001). Conversely, Flores-Moreno et al. (2013) found anemochory and hydrochory dispersal syndromes to be strongly associated with nonnative rather than native species in a global assessment. Discrepancies between our study and their global assessment could be due to the nature of the industrialized flora studied here, again stressing the importance of differentiating between comparing the traits of native and nonnative species in natural versus industrialized or human-dominated sites and among different habitats and environmental conditions more broadly (Milanović et al. 2020).

Nonnative species only showed a significant, positive association to multiple pollination syndromes (Table 1). In general, pollination syndrome is an important trait determining the invasive potential of plants (Gassó et al. 2009), with previous studies finding a variety of pollination syndromes associated with nonnative species, including wind (Andersen 1995), animal (Williamson and Fitter 1996), and self-pollination (Milanović et al. 2020). Similarly, our findings suggests that instead of having one particular pollination syndrome, having multiple pollination syndromes may better benefit the introduction of nonnative species at our study site. Recently, we documented previously undetected hitchhiking species on the air-intake grilles of refrigerated shipping containers and primarily found wind-dispersed, nonnative graminoids entering the Port of Savannah via maritime trade (Lucardi et al. 2020b). Though not all of these inadvertently moved propagules will result in plant establishments, the intensity of the propagule pressure at this industrialized site further underscores the immediate need to target such areas with stronger prevention and interception programs and extensive EDRR (Burk 1877; Lucardi et al. 2020b).

As highlighted above, we found differences in the traits associated with native versus nonnative species, notably, significant differences in associations of nativity with pollination syndrome and duration (Table 1). This trend contrasts with previous research that found no difference in species' traits with different nativity (Thompson et al. 1995; Leishman et al. 2010; Tecco et al. 2010). Pyšek et al. (1995) did not find a significant difference between pollination syndromes of native and nonnative species in

a Czech flora. Still, they did see a significant difference in dispersal agents. Importantly, the findings of Pyšek et al. (1995) were only apparent when native and nonnative species were examined in anthropogenic habitats compared to natural environments. The findings of Pyšek et al. (1995) and Wolf et al. (2020) highlight the context-dependency associated with the influence of human-dominated systems, such as industrialized and urban floras, and the uniqueness of the species in these floras as compared to natural sites. For instance, the flora analyzed in this study is composed of approximately 35% nonnative species, a higher percentage than found in 19 other comparison floras published since 1990 in Georgia and South Carolina, USA (Lucardi et al. 2020a). Interestingly, a recent study introducing the Biotic Novelty Index (Schittko et al. 2020) showed that biotic novelty increased due to increasing nonnative species and increasing impervious surfaces, both of which reflect the situation at industrialized sites like the green spaces of the GCT of the Port of Savannah.

We also found phylogenetic signals for all four traits assessed in this study. In other words, closely related species shared more similar traits more often than expected by chance in the industrialized flora at the Garden City Terminal at the Port of Savannah (Table 2). These findings are not surprising given the wide distribution of nonnative species in the phylogeny of this flora, each with many close native relatives (Suppl. material 3). However, we found that native and nonnative species had different traits with different phylogenetic structure patterns at this site (Tables 1, 3). Anemophily pollination syndrome and polychory dispersal syndrome were the only trait categories where both native and nonnative species had significant phylogenetic structure. Both native and nonnative species showed significant phylogenetic clustering for anemophily, suggesting that the environment of this industrialized site favors species with anemophily over other pollination syndromes. Polychory was significantly phylogenetically over-dispersed for native and nonnative species, suggesting species may specialize in different dispersal syndromes that allow them to partition niche space at this site.

Overall, the categories of traits showing a significant phylogenetic structure in the flora, for the most part, differed from those that had significant relationships with species nativity (Table 1). Only traits of native species (zoophily pollination syndrome and perennial duration) showed significant associations with species nativity (native) and also had non-random patterns of phylogenetic structure (clustering). These results suggest a strong tendency of native species to have perennial duration and zoophily pollination syndrome, which may reflect the regional species pool and deserves future investigation.

Determining what makes communities invasion-prone has been elusive. There are intuitive arguments for environmental filtering, whereby nonnative plants should have traits similar to native ones, and empty niche or niche partitioning, whereby nonnative plants should have different traits from native ones (Elton 1958; Bezeng et al. 2013; Hulme and Bernard-Verdier 2018; Enders et al. 2020). Our findings suggest that both mechanisms are in play at this industrialized study site. Phylogenetic structure patterns and differences in the associations of traits among native

and nonnative species suggest that niche partitioning facilitates the introduction and survival of nonnative species at our site (MacDougall and Turkington 2005). Complementing these differences are traits of nonnative species commonly associated with species in highly disturbed industrialized or urban floras, including annual duration, variation in pollination and dispersal syndromes (Williams et al. 2015; Palma et al. 2017). The environment of the industrialized flora at the Port of Savannah may be filtering for species with these traits. Based on findings in previous research, the introduction of native species at this site may have also been influenced by the environment as successfully occurring species show adaptation to weedy and disturbed habitats (Lucardi et al. 2020a). Similar findings to this study have been categorized as species needing similar traits when introduced to a novel community (environmental filtering) but also requiring distinct traits to disrupt the community (niche partitioning), as invaders are notoriously known for doing (Ordóñez 2014; Divíšek et al. 2018). In the future, environmental filters associated with this human-dominated, highly disturbed site may further limit the traits and phylogenetic distribution of species within this site which may create a habit even more conducive to the introduction and survival of nonnative species (Williams et al. 2009; Bennett et al. 2014).

Despite the small amount of green space at the Garden City Terminal (~1% of the land area at the industrialized site), this industrialized flora is unique with a large number and proportion of nonnative species (Lucardi et al. 2020a). Many studies assessing species' traits in other floras have focused on other traits not available for most of the species in this study, limiting our ability to make a comparison with their findings. We also recognize that lower phylogenetic resolution may contribute to uncertainty in the phylogenetic analyses (Swenson 2009). However, 61% of branches in the phylogeny were highly supported (bootstrap support > 85%) and 68% were moderately supported (bootstrap support > 70%). In the future, additional DNA barcode regions would better help resolve the phylogeny. In the phylogenetic analyses, significant phylogenetic patterns were found for either MPD or MNTD, but not both metrics in some cases. These discrepancies may be due to the nature of these metrics, where MPD captures the overall phylogenetic dissimilarity of species in the group, whereas MNTD can detect finer scale phylogenetic patterns at the tips of the phylogeny that may be present (Erickson et al. 2014; Swenson 2014).

Conclusions

This research highlights differences in duration along with pollination and dispersal syndromes associated with species nativity that deserve consideration and further investigation in future studies of industrialized floras. It also highlights, through phylogenetic analyses, how highly disturbed sites may filter for species with traits such as anemophily pollination syndrome, regardless of species nativity. Finally, this research suggests the influence of environmental filtering and niche partitioning on the

similarity and dissimilarity, respectively, of nonnative and native species traits that may have allowed their introduction and survival at this site. More research, cooperation, and coordination are needed at industrialized and urbanized sites to more adequately investigate nonnative species' establishment and spread probabilities (Tsang et al. 2019; Lucardi et al. 2020b; Borden and Flory 2021). Regular monitoring at these sites is also essential for documenting spatiotemporal changes in plant and trait communities that favor the establishment of nonnative species through localized population dynamics and the hosting of newly arriving propagules.

Data availability

The GenBank accession numbers for all successfully sequenced *rbcl* and *matK* DNA barcodes can be found in Suppl. material 2. Sequences are also publicly available on the Barcode of Life Data System (**BOLD**). Alignments used to generate the phylogenetic tree and the phylogeny in Suppl. material 3 along with all code can be found at <https://doi.org/10.5281/zenodo.7101888>.

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Author contributions

SJW, TDM, RDL, and KSB were involved in the conception of the idea. SJW, LEW, RDL, and KSB collected data. TDM identified the species morphologically. LEW prepared specimens for DNA barcode analysis, SJW analyzed data, and SJW wrote the original draft with edits, comments, and approvals from all authors.

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Supplementary material I

Species included in this study, listed alphabetically, along with their traits

Authors: Samantha J. Worthy, Travis D. Marsico, Rima D. Lucardi, Lauren E. Whitehurst, Kevin S. Burgess

Data type: docx file

Explanation note: Species included in this study, listed alphabetically, along with their traits. All species are represented by vouchers stored at Arkansas State University Herbarium (STAR) and Columbus State University Herbarium (COLS). If citations are not included, the information was gathered from the USDA PLANTS Database (USDA, NRCS, 2021).

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Supplementary material 2

Sequencing and collection information for the species in this study

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Data type: docx file

Explanation note: Sequencing and collection information for the species in this study, listed alphabetically. The collection number and GenBank accession number for each sequence are presented below. Sequences downloaded from Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007) and GenBank (Benson et al. 2013) databases are noted with a * by the accession number. Only species with both DNA barcode gene regions were included in phylogenetic analyses, eliminating nine species that had either only a *rbcL* or *matK* sequence, noted by “Excluded” in the GenBank Accession column. Sequences for two species placed them into incorrect areas of the phylogenetic tree causing us to question their identity and eliminate them from phylogenetic analyses, noted as “Erroneous Sample” in the GenBank Accession column. Failed sequencing is noted in the sequence column and no GenBank accession number is given. All species are represented by vouchers stored at Arkansas State University Herbarium (STAR) and Columbus State University Herbarium (COLS).

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Supplementary material 3

Phylogenetic tree depicting genetic relationships among 159 species of the flora at the Port of Savannah, Savannah, Georgia, USA, with available sequences

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Data type: docx file

Explanation note: Phylogenetic tree depicting genetic relationships among 159 species of the flora at the Port of Savannah, Savannah, Georgia, USA, with available sequences. The phylogeny was generated using maximum likelihood methods in the “phangorn” package (Schliep 2011) in R programming language (R Core Team 2021) using the general time-reversible model of substitution with gamma-distributed rate variation and a proportion of invariant sites (GTR + Γ + I) with *Ginkgo biloba* as the outgroup. Bootstrap values from 1000 replications are presented at each node. Tree topology was visualized using iTOL v. 6.3 (Letunic and Brok 2021). Species in black font are native, and species in blue font are nonnative.

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Genetic divergence and aggressiveness within a supercolony of the invasive ant *Linepithema humile*

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Abstract

Biological invasions constitute an opportunity to study the evolutionary processes behind species' adaptations. The invasive potential of some species, like the Argentine ant (*Linepithema humile*), has likely been increasing because they show low intraspecific competition. However, multiple introductions over time or genetic divergence could increase the probability of intraspecific competition, constituting barriers for their dispersal and thus, decreasing invasive success. Here, we studied the genetic and behavioural variability of *L. humile* workers collected at six locations on the NW coast of the Iberian Peninsula, a possible scenario for multiple introductions and population divergence, due to its high level of maritime traffic and complex coastal geography. We analysed behaviours related to spatial navigation (exploration, wall-following), resources acquisition, and competition (inter and intraspecific aggressiveness) through two relevant seasons for the nest ecology: spring and autumn. Genetic analyses using microsatellites indicated that the nests studied belonged to the most spread supercolony in South Europe. However, we identified the existence of two genetically differentiated clusters in Galiza. Lethal interactions were found between workers from different and similar genetic clusters, but a trend suggests higher agonistic behaviours between the two genetic groups. Genetic differences were positively correlated with the geographical distance, but aggressiveness was not correlated with any of them. Ants from each of the tested nests expressed different behaviours with high plasticity through time. Ants from all nests showed more exploration and aggressiveness, less wall-following and faster detection of food in autumn than in spring, with no intraspecific aggressiveness observed in spring. Our findings suggest competition between nests of the same supercolony

and behavioural seasonal variability, supporting the hypothesis of divergent evolutionary processes. The results of our work question the assumed unity of supercolonies of this species and offer insights for understanding the future adaptation of *L. humile* in the introduced areas.

Keywords

intraspecific competition, population genetics, subcolony, unicoloniality, variability

Introduction

The spread of exotic invasive species constitutes one of the most serious threats to biodiversity (Dueñas et al. 2021), bringing with it high economic impacts (Bradshaw et al. 2021; Haubrock et al. 2021; Angulo et al. 2022). The introduction of exotic invasive species has greatly increased in the last decades due to market globalization and its synergy with climatic change (Galil et al. 2007; Perrings et al. 2010; Ramsfield et al. 2016). For invertebrates, traits like their small body size, large population numbers, or high reproductive potential hinder the application of control programs (Hoffmann et al. 2016) and therefore, many exotic invertebrates become pests in their introduced areas. In particular, social insects are among the organisms with the highest representation and the greatest threat to ecosystems (Siddiqui et al. 2021). Invasive ants displace native species, alter ecosystem services (food web, recycling, pollination), protect species considered as pests (e.g. aphids), invade human installations, and transmit pathogens to other species, causing ecological costs valued at US\$ 10.95 billion over the last 90 years (reviewed in Pedersen et al. 2006; Baty et al. 2020; Angulo et al. 2022).

One of the most worrying points in conservation programmes, albeit one that is extremely interesting for science, is the evolution of the introduced species (e.g. Eurohornet project; Wystrach and Lihoreau 2020). Invasive species succeed in adapting to newly colonized environments in a very narrow window of time, especially in human dominated ecosystems (Pyšek et al. 2010), usually developing higher fitness than native species (Boltovskoy et al. 2020). The evolution of introduced-invasive ants allows us to record complex evolutionary processes at a human time scale as local adaptation and intraspecific divergence (Helanterä 2022), and to understand the species trade-offs, which could constitute a key factor in the fight against the ecological and economic problems that these species cause.

One of the most relevant examples of invasion due to social organization emerged between 1882 and 1891 with the introduction of the Argentine ant (*Linepithema humile* Mayr, 1868) into Madeira and New Orleans (Newell and Barber 1913; Suarez et al. 2001). This first introduction event was followed by others which quickly spread this species around the world (Suarez et al. 2001). The success of *L. humile* is related to a change in its intraspecific competition. In its native distribution, *L. humile* colonies are composed of connected nests extending up to 500 m ca. (unicolonial supercolonies; Pedersen et al. 2006). Although there is no evidence of individual or resources flow between long distanced nests (Moffett 2012), individuals do not compete or attack

when belonging to the same supercolony while they show high aggressiveness towards members of other supercolonies (Pedersen et al. 2006). However, only a small number of vast supercolonies extend over thousands of kilometres in the introduced areas (e.g. Giraud et al. 2002; Thomas et al. 2007; Blight et al. 2010). The way in which this new ecological scenario has emerged is still under debate, although it is assumed to be the product of a colony founding and genetic bottleneck (Suarez et al. 1999) possibly followed by the dominance of colonies with reduced genetic diversity (Tsutsui et al. 2003) or selection pressures under high densities (Giraud et al. 2002; but see Thomas et al. 2006). The invasive potential of these vast supercolonial organisations (Holway et al. 1998) resides in their capability for unrestricted growth (Moffett 2012), thus making *L. humile* one of the most dangerous introduced species (Lowe et al. 2000). Even if it is possible to reduce its presence in the introduced areas (Tatsuki et al. 2012; Hoffmann et al. 2016; Angulo et al. 2019), most authors agree with the impossibility of its eradication and therefore, only preventive methods to fight against the expansion of this species can be proposed (Siddiqui et al. 2021).

Although it was proposed that the introduction of new supercolonies would not interfere with the expansion of well-established supercolonies (Moffett 2012), the existence of several supercolonies in the same geographic area leads to resource limitation and the death of millions of individuals per year at the border between supercolonies (Thomas et al. 2007). Therefore, the emergence of new supercolonies may induce intraspecific competition – the missing characteristic of the introduced supercolonies – and reduce the species environmental effects (Moffett 2012; Helanterä 2022). The success of the invasive potential of *L. humile* could be (theoretically) disrupted by two main processes: (i) multiple introductions of native colonies that maintain competition in the introduced area (e.g. Buczkowski et al. 2004; Vogel et al. 2009) and (ii) the divergent evolution of colonies already established in the introduced areas caused by local adaptations (see Ingram 2002; Moffett 2012; Helanterä 2022) and the lack of genetic flow between colonies (see Heller 2004; Pedersen et al. 2006). We did not consider the foundation of new supercolonies by flying queens of the established supercolonies due to the unexpectedness of this process (Markin 1970; Helanterä 2022). In the first case, assuming *L. humile* is introduced via maritime flow, a high number of competitive colonies would be expected in coastal regions with commercial harbours and high genetic diversity might be expected in these areas (see Moffett 2012), as happens on the Californian coast (Suarez et al. 2002; Thomas et al. 2007). In the second case, however, lower competition between colonies would be expected due to the dependence of divergent evolution on long-term isolation processes. This process was suggested as the cause of emergence of new supercolonies in Europe (Moffett 2012) due to the similar genetic diversity, chemical cuticular composition, and low aggressiveness occurred between the Corsican supercolony and the main supercolony (Blight et al. 2010). In both cases, variability in behaviour between colonies may be expected, with higher variability in the case of adaptation of native colonies to newly colonized environments (multiple introductions) than in the case of local environmental adaptations of previously established colonies (evolutionary divergence).

Linepithema humile shows behavioural variability both in its native and introduced areas (Blight et al. 2017) and in the latter, also among (Giraud et al. 2002; Blight et al. 2010) and within supercolonies (Chen and Nonacs 2006; Thomas et al. 2007; Van Wilgenburg et al. 2010a). As workers' phenotypic plasticity conditions the colony behaviour (Pinter-Wollman 2012), local environmental differences may lead to local adaptations and thus, to divergence and competition (see Helanterä 2022). Behavioural divergence due to differences in spatial location might increase variation in colony productivity or food consumption, both factors that increase variation in cuticular compounds (Liang and Silverman 2000; but see Giraud et al. 2002; Buczkowski et al. 2005; Walsh J et al. 2020) and therefore, act as modifiers of the interactions between individuals from distanced nests of the same supercolony.

Seasonality constitutes a further environmental factor able to modify ant colony behaviour (Markin 1970; Benois et al. 1973; Thomas et al. 2005, 2006, 2007; Heller and Gordon 2006) and individual cuticular compounds (Abril et al. 2018), possibly leading to changes in the ants' invasive potential (Thomas et al. 2006). Understanding how locally adapted nests deal with seasonal changes would help to decipher present divergent responses but also the future scenario modified by the effect of climatic change. In a similar way, environmental changes due to climatic trends could modify the costs and benefits for invasive species and open new distribution areas (Thomas et al. 2006).

In this study, we focus on ethological and genetic analyses of *L. humile* workers collected at six different localities in Galiza (NW Iberian Peninsula), an area where the biology of the species is poorly known (Giraud et al. 2002; Gómez and Espadaler 2004). Galiza constitutes a relevant point of maritime commercial flow between South America and Europe, increasing the likelihood of the introduction of exotic species and the establishment of colonies of multiple origin (Castro et al. 2017). If multiple introductions of *L. humile* from its native range have occurred in Galiza, we would expect to find a high number of well-limited genetic and behavioural groups. Galiza's coastal configuration is characterized by estuaries and habitat diversity, which could favour allopatric and sympatric events respectively. If the nests of the main supercolony (the supercolony present in this region; see Giraud et al. 2002) suffered local adaptations and allopatry, we would expect a progressive genetic and behavioural diversity. To test these hypotheses, we carried out a set of behavioural tests in six sampled nests of *L. humile* from Galiza, similarly to previously published works (Blight et al. 2017). We controlled for seasonal effects by studying the behaviour of workers from the studied nests after and before winter. To verify if the lack of aggressiveness between conspecifics of the different nests studied was due to general loss of aggressiveness (for instance, linked to seasonal effects; Ichinose 1991), we analysed the aggressiveness of *L. humile* against a native species (*Myrmica rubra* Linnaeus, 1758). Finally, to analyse the genetic variability and population structure within our study area, we genotyped individuals from the nests sampled in Galiza, together with samples belonging to the two supercolonies previously identified in the Iberian Peninsula (Giraud et al. 2002) at seven microsatellite loci.

Materials and methods

Specimen collection

Individuals from one ant nest were collected in March and September 2020 at each of four locations (Carril Garden, Ribeira, Pontevedra and Reboreda), distanced by approximately 30 km on a latitudinal N-S axis following the Galizan Southern coast (see Fig. 1; Table 1). In a preliminary test performed in the laboratory in August 2019 with marked ants, workers belonging to Carril Garden killed 100% of workers from Pontevedra ($N = 20$). Given these results, we aimed to determine if the high aggressiveness showed by individuals from Carril Garden could be also expressed in other nests geographically close; or if this was a characteristic of this particular nest. To this aim, we collected individuals from two nests located at approximately 1–2 km from Carril Garden (Carril Coast and Trabanca). We assumed each nest was maintained in the same place across seasons (see Vogel et al. 2009), but we did not analyse the flow of individuals between nests. Ants were collected using small shovels and brushes.

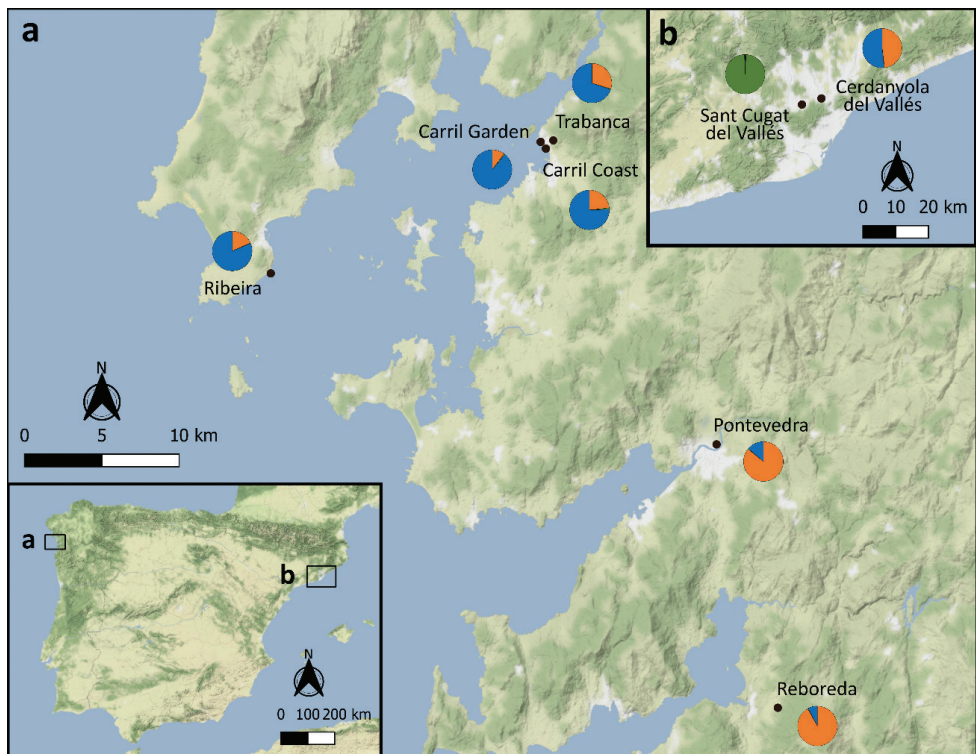


Figure 1. Map showing the location (black dots) of the eight colonies of *Linepithema humile* sampled for this study and listed in Table 1. Pie charts by each locality illustrate the proportion of individuals from each locality that were assigned to each of the genetic clusters identified by STRUCTURE (see Results and Fig. 7 and Suppl. material 1: Fig. S3). The figure has been created using QGIS version 3.22.3 (Anon 2022).

Table 1. Details on the *Linepithema humile* colonies sampled for this study shown in Fig. 1. Listed are the locality name, the type of environment and the coordinates for each sampled population. The order in which the colonies sampled in Galiza are listed follows a N-S latitudinal gradient (see Fig. 1). For the two localities sampled in Catalunya, the acronym in brackets refers to whether they belong to the Catalanian (CS) or the main supercolony (MS).

Region	Locality	Environment	Coordinates
Catalunya	Sant Cugat del Vallés (CS)	garden	41°28'29"N, 2°04'39"E
	Cerdanyola del Vallés (MS)	garden	41°29'29"N, 2°08'54"E
Galiza	Trabanca	agriculture	42°36'56"N, 8°45'55"W
	Carril Garden	garden	42°36'52"N, 8°46'29"W
	Carril Coast	coast	42°36'39"N, 8°46'16"W
	Ribeira	coast	42°32'19"N, 8°59'12"W
	Pontevedra	garden	42°26'23"N, 8°38'14"W
	Reboreda	garden	42°17'14"N, 8°35'21"W

Fluon-coated open plastic boxes (18 × 18 × 8 cm) were used to transport the ants to the laboratory. Ants were maintained in the same plastic boxes used for collection, with soil from their own nests (following Giraud et al. 2002) and tubes filled with water and cotton covered with paper as artificial nests. Food (based on Bhatkar and Whitcomb 1970) was provided *ad libitum*. Nests were kept in the laboratory at room temperature, with a photoperiod of 12:12 h. Additionally, samples belonging to the two *L. humile* supercolonies identified in the Iberian Peninsula (Giraud et al. 2002) were collected on 10 March 2021 in Catalonia (Cerdanyola del Vallés and Sant Cugat del Vallés; see Table 1), to be used for the genetic analyses.

Exploration and thigmotaxis tests

In each season, spring (March) and autumn (September), fifty workers from each nest were randomly collected from the outside and inside of the tubes from the experimental nests to avoid biased selection of workers (foragers and nurses). Experimental workers were individually placed in Fluon-coated Petri dishes (Ø = 5.5 cm). Ten minutes of free walk observation were recorded with a Canon Legria HF M56 video camera. Tests were performed daily at 10:00 a.m. for six days. From the videos, we extracted the position of the individuals at a frame rate of 2.08 frames per second using the software Swistrack version 4.0 (Correll et al. 2006). Data were then analysed with R version 4.0.2 (R Core Team 2021) to measure the proportion of time individuals spent moving as a proxy of exploration (locomotor activity under non-risky and novel environments; Réale et al. 2007). An individual was considered as moving if it moved more than 1 mm between two frames. We selected the frequency of movements, rather than the total distance covered by each individual, to avoid effects related with ant body size. We also calculated the time spent far from the dish border (5 mm) as a proxy of border avoidance (i.e. the opposite to wall-following; also see Sanmartín-Villar and Jeanson 2021). Ants navigate their environment by contacting structures (Dussutour et al. 2005), so abandoning the spatial reference might be linked with cognitive performance (Doria

et al. 2019) or personality traits as boldness (Valle 1970; Walsh and Cummins 1976; Sneddon 2003; Carlson and Langkilde 2013; Detrain et al. 2019), factors that might affect individuals' dispersion.

Foraging efficiency test

Two hundred workers from each nest were randomly collected (see above) and placed in groups of 10 in 20 Fluon-coated Petri dishes ($\varnothing = 13$ cm) connected with another Fluon-coated Petri dish ($\varnothing = 5.5$ cm) by a plastic bridge ($5.54 \times 2.51 \times 1.1$ cm). A tube containing water and covered with paper was added to the bigger dish. The focal ants were kept for one day in this experimental setup to get familiarised with the new arenas and to experience a similar period of starvation to standardise their food requirements and thus, their feeding drive. After 24 h, we added food to the small dish. We video recorded the first 30 minutes after adding the food and took pictures of the small dishes 10 min after we stopped recording. We analysed the time needed for the first worker to reach the food and how many individuals were inside the dish containing food after 40 min as a proxy for foraging efficiency (hereafter, "number of foragers"). Tests were performed in March and September at 10:00 a.m. over three consecutive days, testing workers of two nests per day.

Aggressiveness test

To test the aggressiveness between different nests, 100 individuals were randomly collected from each nest and paired with an individual from another nest ($N = 20$ for each possible pair's combination). This procedure was performed in both seasons. Pairs were placed in Fluon-coated Petri dishes ($\varnothing = 5.5$ cm). The time that elapsed between the introduction of the first and second individual was approximately one second. To avoid the residency effect (Shreeve 1987; Peixoto and Benson 2012; but see Kemp and Wiklund 2004), we alternated the order of each individual's addition. Control pairs consisted of two workers from the same nest ($N = 10$ replicates in March, $N = 20$ in September). To analyse the seasonal effect, workers from the same nest but collected at different times (March and September) were paired in September ($N = 220$; 20 per season and nest except for Carril Garden nest, in which no individuals collected in March survived). We used the same procedure to confront workers from each nest with *Myrmica rubra* workers. Interactions within individual pairs were analysed by video recording the first 10 min after the individuals' addition. The video allowed us to identify individuals' nest origin without marking them by following them until the first attack or until the end of the video. We recorded the presence or absence of bites between paired ants on each dish as an indicator of aggressiveness. We compared aggressiveness between nests by comparing the number of dishes where we had observed attacks. We did not record the time engaged on each attack (see Hakala et al. 2020) or posterior attacks produced in the same dish - only the first attack was recorded on each pair - to avoid considering behaviours conditioned by the first attack or repeated measures

(experiences). We did not study other interactions (antennation, touch, trophallaxis, avoidance, gaster elevation) because previous works that considered these ended up merging their score values (Carlin and Hölldobler 1986; Holway et al. 1998; Giraud et al. 2002; Roulston et al. 2003; Vogel et al. 2009; Blight et al. 2012, 2017; Hakala et al. 2020) or interpreted them as binomial interactions (i.e. aggressive or non-aggressive; see Suarez et al. 1999, 2002; Tsutsui et al. 2000, 2003; Thomas et al. 2005, 2006, 2007; Wetterer and Wetterer 2006; Blight et al. 2010). We considered that only measuring attacks is a conservative method that, even if it underestimates the behavioural response during interactions, reflects the agonism of the encounter and alleviates artefacts (for instance, stress produced by handling; Bernadou et al. 2018) and subjective interpretations. Paired individuals were maintained for 24h with wet cotton to check mortality (Blight et al. 2010).

Observers were distanced from the ants when performing all behavioural tests described above and wore gloves and masks when manipulating the individuals to avoid impregnating body waxes and exhaling in their direction (Chen and Nonacs 2006). We assumed ant manipulation did not elicit aggressiveness (Heller 2004; Bernadou et al. 2018) or if it occurred, we assumed the same effect for all workers tested.

DNA extraction, PCR, and microsatellite genotyping

Total genomic DNA was extracted from 24 workers from each nest using the GeneJet DNA extraction kit (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. Genetic variability was assessed by using seven microsatellite polymorphic loci: *Lihu-S3*, *Lhum-11*, *Lihu-T1*, *Lhum-13*, *Lhum-19*, *Lihu-M1* and *Lhum-62* (Krieger and Keller 1999; Tsutsui et al. 2000). PCRs were carried out in a total 10 µl volume containing 5 µl of DreamTaq Green PCR Master Mix (ThermoFisher Scientific), 0.2 pmol of each primer, and ~10 ng of genomic DNA. Cycling conditions consisted of 5 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at T_a °C and 30 s at 72 °C, and a final extension of 2 min at 72 °C (where T_a is the marker-specific annealing temperature; see Suppl. material 1: Table S1). PCR products were pooled into one of two genotyping panels, depending upon the expected allele sizes and the 5' fluorescent dye (6-FAM, NED, PET or VIC; Applied Biosystems, Foster City, CA, USA); along with GENESCAN-500 LIZ size standard (Applied Biosystems). Products were separated using capillary electrophoresis on an ABI3130 automated DNA sequencer (Applied Biosystems) at the CACTI genomics facility from the University of Vigo. Allele bins and sizes for each locus were determined with the 3rd order least squares method implemented in the microsatellite plugin from Geneious version 9.1.8 (Kearse et al. 2012).

Genetic diversity and population structure analyses

Observed and expected heterozygosities, the number of private alleles for each locus and deviations from Hardy-Weinberg equilibrium (HWE) for each locus in each

location were calculated using ARLEQUIN version 3.5 (Excoffier et al. 2005). The Bayesian model-based clustering approach implemented by STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to identify genetic clusters while assigning individuals to each of these clusters. STRUCTURE analyses were carried out including all the genotyped localities (i.e., Catalanian and main supercolony samples plus the nests sampled in Galiza), in order to determine whether the samples of Galiza belonged to the main or the Catalanian supercolony. We used the admixture model, and the number of clusters (K) was estimated by comparing the log-likelihood ratios in two independent runs for values of K between 1 (panmixia) and 8 (the total number of sites sampled). Each run consisted of 5^6 iterations, with a burn-in period of 5^5 iterations, to ensure convergence of the Monte Carlo Markov Chain (MCMC). A second run of STRUCTURE was carried out to analyse the genetic structure within samples from the main supercolony (i.e., excluding Sant Cugat del Vallés) as determined from the previous analysis; with four independent runs for values of K between 1 and 3. The scale of major population subdivision within our datasets (i.e. the value of K that maximizes the posterior probability of the data) was calculated following the method of Evanno et al. (2005), with ΔK calculated using STRUCTURE HARVESTER (Earl and vonHoldt 2012; available at <http://taylor0.biology.ucla.edu/structureHarvester/>).

Pairwise F_{ST} values were calculated in ARLEQUIN version 3.5 (Excoffier et al. 2005), using the number of alleles distance method and with significance assessed by 1,000 permutations. Additionally, we calculated the level of genetic similarity between colonies as the percent of shared alleles (i.e., the number of alleles shared across loci between localities/total number of alleles possessed by both localities; as in Tsutsui et al. 2000).

Geographical distances

The geographical distances between the nests sampled in Galiza were estimated using QGIS version 3.22.3 (Anon 2022), either by measuring the shortest distance (beeline, considering ants displacement by the sea) or by measuring the length of the shortest terrestrial path connecting colonies. The two methods differ mainly in the consideration of the location of the Ribeira nest.

Statistical analyses

Statistical analyses were performed using R version 4.0.2 (R Core Team 2021). We ran linear models (LMs) using exploration (proportion of time spent moving) or lack of thigmotaxis (proportion of time spent out of the arena edge) as response variable, while nest and season were selected as fixed factors in the analyses. Nests were compared by pairs in *post hoc* analyses using the *emmeans* package (Lenth 2021). We compared the time needed to reach the food for the first ant of each replicated group in the foraging tests using survival curves with the Kaplan-Meier method (Kassambara et al. 2021). The total number of foragers present in the feeding dish after 40

min of food addition and aggressiveness (total number of ants performing the first attack) were analysed for each season by contingency chi-squared tests with Holm's correction. For the tests of aggressiveness, the relationship between the number of pairs with aggressions and the genetic similarity (percentage of shared alleles) or the geographic distances (both beeline and terrestrial) were assessed with a generalised linear model (GLM) with binomial data and logit link function. Proportions of pairs with aggressions was the response variable and the three different distances were fixed factors. Interactions between genetic and both geographic distances were considered but we removed them due to the lack of significance. We used F_{ST} values, which are the proportion of the total genetic variance contained in a subpopulation (S) relative to the total genetic variance (T) (Wright 1949), as a measure of the degree of genetic differentiation between nests. To have a general view of the nests behavioural differences between seasons, we carried out principal component analyses (PCAs) for all behaviours measured (exploration, thigmotaxis, first forager, number of foragers, and interpopulation and interspecific aggressiveness) by using the *prcomp* function (stats package).

Data availability

Data are provided as supplementary information. Information on genotypes for the sampled populations is available upon request to the authors.

Results

Exploration and thigmotaxis tests

Workers' movement frequency differed among the six studied nests ($F_{5,587} = 7.28$, $p < 0.001$; Table 2), between seasons ($F_{1,587} = 398.36$, $p < 0.001$), and there was an interaction Nest \times Season ($F_{1,587} = 6.70$, $p < 0.001$, $R^2 = 0.44$; Fig. 2A). Workers from all nests increased their exploration in autumn (differences ranging between 19% up to 42%, $p < 0.001$).

The use of the border by workers differed among the six studied nests ($F_{5,592} = 2.64$, $p = 0.022$, $R^2 = 0.08$; Fig. 2B; Table 2). Workers used more the edge of the arena in spring ($F_{1,592} = 40.96$, $p < 0.001$), but no significant differences were found for the interaction Nest \times Season ($F_{5,587} = 1.66$, $p = 0.142$).

Foraging efficiency test

Workers' first arrival at the food differed among nests (spring: $\chi^2 = 23.4$, $df = 5$, $p < 0.001$, Fig. 3A; autumn: $\chi^2 = 14.1$, $df = 5$, $p = 0.020$, Fig. 3B; Table 2). Workers reached the food faster in autumn than in spring (9.61% in average; $\chi^2 = 10.1$, $df = 1$, $p = 0.001$, Fig. 3C).

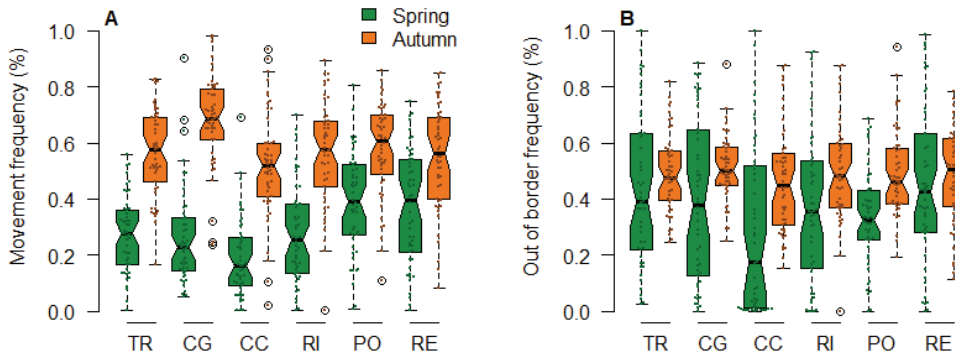


Figure 2. Frequency of movement (exploration **A**) and out of border frequency (proportion of time in which the ants did not use the arena border; thigmotaxis **B**) showed by each of the colonies of *Linepithema humile* from Galiza included in this study, in both spring and autumn seasons. The horizontal line in each box represents the median, and the lower and upper hinges indicate the first and third quartiles. Lower and higher whiskers extend to the most extreme values within 1.5 interquartile ranges from the first and third quartiles, respectively. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

The number of workers reaching the food 40 minutes after its addition was different among nests (spring: $\chi^2_5 = 46.09$, $p < 0.001$; autumn: $\chi^2_5 = 41.73$, $p < 0.001$; Fig. 3D; Table 2). More workers (average 7.73%) were present in the feeding dish in spring than in autumn ($\chi^2_1 = 23.76$, $p < 0.001$).

Aggressiveness test

No fights were observed between paired *L. humile* workers during the 10 min of observations carried out in March. Mortality after 24 h was only found in dishes shared by workers from nests of Carril Coast and Pontevedra (7.5%) and from Trabanca and Pontevedra nests (10%).

In September, no fights were observed between paired workers of the same nest (control) during the 10 min of recorded observations. No fights were observed between individuals from the same nest but collected in different seasons (March and September) in Trabanca and Reboreda, while fights were observed in 10–20% of dishes when mixing individuals from different seasons in Carril Coast, Ribeira, and Pontevedra nests (Fig. 4A). Fights between workers collected in September were observed in all kinds of combinations confronting individuals from different nests, except the cases in which workers from Carril Coast nest were mixed with workers from Ribeira and Reboreda nests. The number of dishes in which attacks occurred depended on the nests pairs' combinations ($\chi^2 = 36.86$, $df = 14$, $p < 0.001$; Fig. 4A). Workers belonging to Carril Garden – Reboreda fought in 50% of pairs, followed by Carril Coast – Pontevedra (25%), Trabanca – Pontevedra and Carril Garden – Pontevedra (20%), Trabanca – Carril Garden and Carril Garden – Carril Coast (15%), Trabanca – Carril

Table 2. Between nests *post hoc* significant differences for all the behavioural variables measured, except aggressiveness. *P*-values correspond to Holm’s corrected *p*-values. $N_{\text{exploration}} = 50$ (except for TR in spring, $N = 49$); $N_{\text{out of border}} = 50$; $N_{\text{arrival to food}} = 10$; $N_{\text{number of foragers}} = 10$. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

Behaviour	Season	Nest pair				<i>p</i> -value
		Nest	Mean±SD	Nest	Mean±SD	
Exploration (%)	Spring	PO	38.67±17.93	TR	27.55±13.31	0.016
				CG	26.43±17.43	0.005
				CC	18.91±14.20	<0.001
				RI	27.93±18.02	0.020
		RE	37.16±20.54	TR	27.55±13.31	0.049
				CG	26.43±17.43	0.020
				CC	18.91±14.20	<0.001
				PO	57.55±16.73	0.030
	Autumn	CG	68.14±16.05	TR	56.76±15.63	0.015
				CC	52.30±18.91	<0.001
				RI	56.18±18.63	0.009
				RE	55.16±18.81	0.004
Out of border (%)	Spring	CG	39.49±29.23	RE	44.49±27.86	0.033
Arrival to food (s)	Spring	CG	13.12±10.56	TR	4.80±3.06	0.021
				RE	4.69±2.14	0.012
		RI	13.08±9.92	TR	4.80±3.06	0.001
				RE	4.69±2.14	0.001
Number of foragers	Spring	RE	3.65±1.57	CG	1.45±1.50	<0.001
				CC	1.75±1.55	<0.001
				RI	1.25±1.45	<0.001
		TR	2.55±1.57	RI	1.25±1.45	0.017
				CG	0.95±0.89	<0.001
				CC	1.45±1.47	0.024
	Autumn	TR	2.75±1.83	RI	0.80±0.77	<0.001
				PO	1.05±2.09	<0.001
				RE	1.35±0.99	0.001
				CG	0.95±0.89	<0.001

Coast, Trabanca – Ribeira, and Pontevedra – Reboreda (10%), Trabanca – Reboreda, Carril Garden – Ribeira, Ribeira – Pontevedra, and Ribeira – Reboreda (5%). The number of attacks observed showed no significant correlation with the percentage of shared alleles or the geographical distances (terrestrial and the shorter distance) between nests ($p > 0.503$; see Fig. 5A, B). A similar proportion of attacks was observed within (9.29%) and between (16.2%) the North and South genetic clusters identified in Galiza ($W = 33$, $p = 0.597$). Workers from both clusters triggered the attack towards workers from the other clusters in a similar way (North = 60.87%, South = 39.13%; binomial test: $p = 0.405$).

Mortality after 24 hours differed among nests ($\chi^2 = 87.48$, $df = 14$, $p < 0.001$; Fig. 4B). The highest number of deaths was observed in pairs confronting Pontevedra with Carril Coast (35%) and Trabanca workers (30%), followed by Reboreda with Carril Garden (20%) and Carril Coast (17.5%). Fewer than 7.5% of workers died

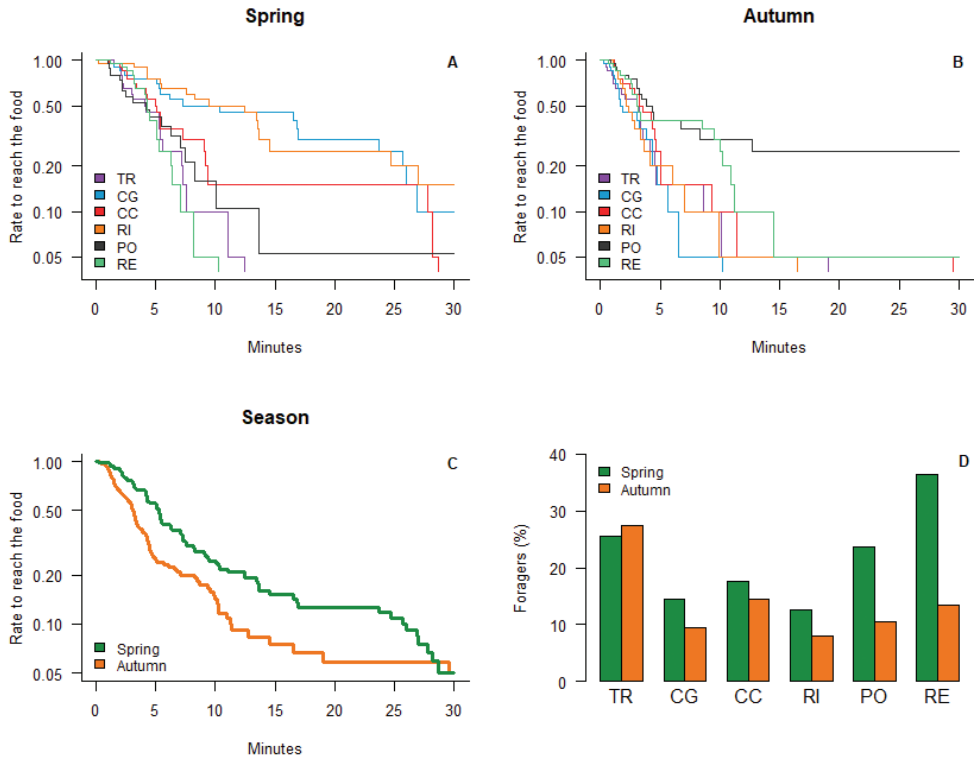


Figure 3. Rate of individuals reaching the food for the first time for each *Linepithema humile* nest sampled in Galiza in spring **A** and autumn **B** overall for each season **C** and proportion of workers from each nest present in contact with the food after 40 min of the food addition **D** Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

in the other combinations and no death events were recorded between Ribeira with Carril Garden or Pontevedra and between Reboreda with Trabanca or Pontevedra. A worker died in the Pontevedra control group in March while four died in September (Trabanca = 1, Carril Garden = 1, Ribeira = 2).

Linepithema humile engaged faster in fights with *Myrmica rubra* (0.73 ± 1.21 min after being paired) than with their conspecifics from different nests (5.43 ± 2.28 min; $t = 12.79$, $df = 69.42$, $p < 0.001$). *Linepithema humile* workers started more fights than *M. rubra* workers when they were mixed in spring (mean *L. humile* = 4.17 ± 2.13 , mean *M. rubra* = 1.00 ± 0.63 ; $t_{5.87} = 3.48$, $p = 0.014$) and autumn (mean *L. humile* = 6.33 ± 2.66 , mean *M. rubra* = 2.33 ± 1.75 ; $t_{8.56} = 3.08$, $p = 0.014$; Fig. 6). No differences between nests or seasons were found for the aggressiveness of *L. humile* towards *M. rubra* and vice versa ($\chi^2 < 8.16$, $p > 0.147$).

Linepithema humile workers died more than *M. rubra* workers when they were mixed in spring (mean *L. humile* = 9.17 ± 3.6 , mean *M. rubra* = 4.50 ± 1.4 ; $t_{6.43} = 2.96$, $p = 0.023$) and autumn (mean *L. humile* = 14.00 ± 1.9 , mean *M. rubra* = 3.83 ± 1.8 ; $t_{9.99}$

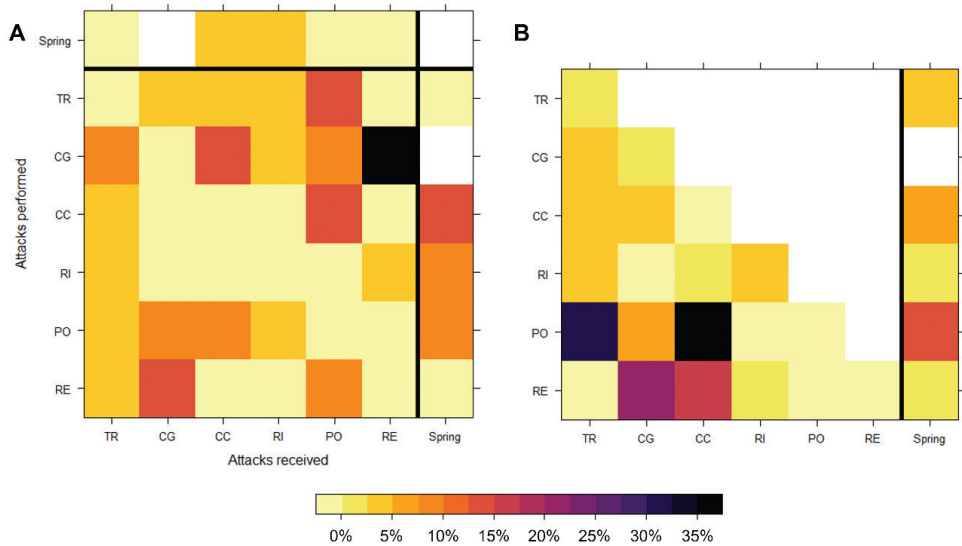


Figure 4. Proportion of attacks performed and received for the first time **A** and mortality **B** of *Linepithema humile* workers collected in Galiza in September towards workers from other nests collected in the same season, workers from the same nest and season (Control), and workers from the same nest but different season (spring). Note that when measuring mortality, nest ID could not be identified and therefore, corpses could belong to any of the two paired colonies. Workers from CG collected in March died before tests performed in autumn. Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

= 9.44, $p < 0.001$; Fig. 6). The mortality of *L. humile* workers confronted with *M. rubra* workers for 24h differed among nests in spring ($\chi^2 = 13.06$, $df = 5$, $p = 0.023$) but not in autumn ($\chi^2 = 4.29$, $df = 5$, $p = 0.509$). The mortality of *M. rubra* workers did not differ according to the *L. humile* nests they confronted in both seasons ($\chi^2 < 5.43$, $p > 0.365$).

According to the PCA results, nests showed no behavioural consistence across seasons (Suppl. material 1: Fig. S1) and thus, no particular nest behavioural pattern could be identified. For example, Reboreda nest showed the highest values of exploration and foraging (first forager and number of foragers) in spring, while the same nest in autumn showed low values for these behaviours. Ribeira and Pontevedra nests showed different behaviour in spring, while individuals from these same nests behaved similarly in autumn.

Genetic analyses

Allelic diversity in the *L. humile* genotyped populations ranged from 1 to 6 alleles per locus, with 38 alleles identified across all 7 loci. Significant deviations from Hardy-Weinberg equilibrium were found at all loci: *Lihu-S3* (Pontevedra); *Lhum-11* (Sant Cugat del Vallés, Pontevedra); *Lihu-T1* (Sant Cugat del Vallés, Cerdanyola del Vallés, Trabanca, Carril Garden, Ribeira, Pontevedra, Reboreda); *Lhum-13* (Sant Cugat del

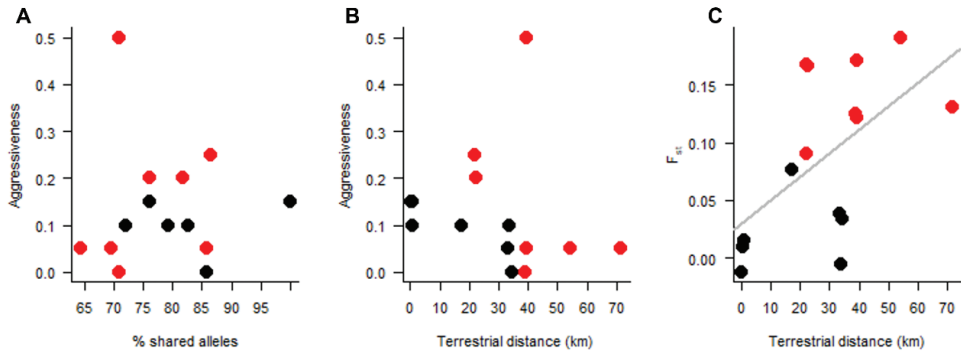


Figure 5. Relationships **A** between the percentage of shared alleles and the aggressiveness **B** between terrestrial distance and aggressiveness; and **C** between terrestrial distance and F_{ST} . Regression line is drawn for significant relationship ($r = 0.57$). Black dots represent pairs of colonies from the same genetic cluster, while red dots represent pairs of colonies from different genetic clusters. Note that these graphs include only populations sampled in Galiza.

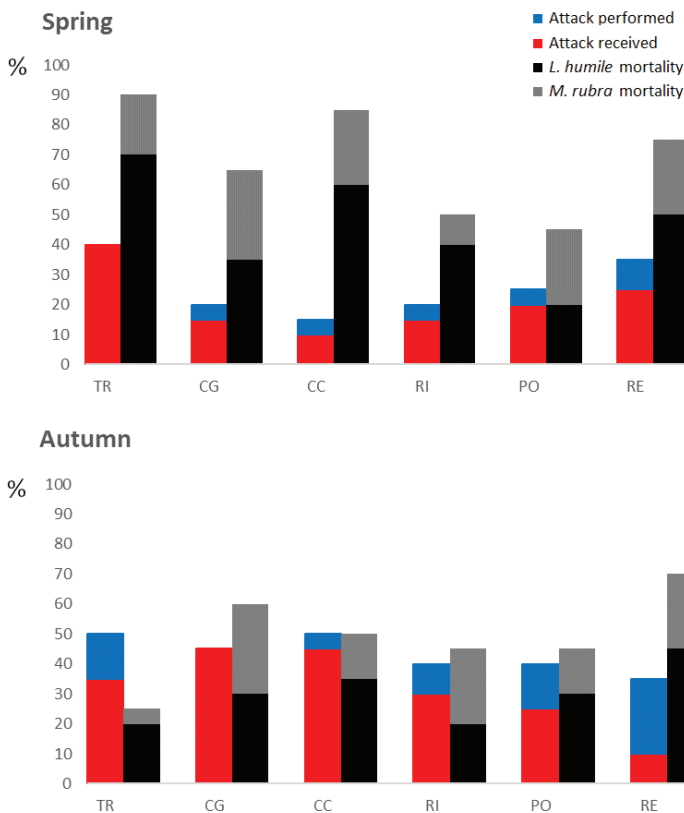


Figure 6. Proportion of attacks performed and received for the first time by *Linepithema humile* from Galiza and mortality after 24 hours paired for *L. humile* and *Myrmica rubra* in spring and autumn. Tra-banca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

Table 3. Summary of genetic diversity for each of the *Linepithema humile* nests sampled for this study. For each locality, we list the mean number of alleles (Na), the mean observed heterozygosity (Ho), and total number of private alleles (Pa) across all seven microsatellite loci used for genotyping listed in Suppl. material 1: Table S1. Detailed information on genetic diversity indexes for each population/locus is provided in Suppl. material 1: Table S2.

Region	Locality	Supercolony	Na	Ho	Pa
Catalunya	Sant Cugat del Vallés	Catalonian	2.33	0.683	7
	Cerdanyola del Vallés	Main	3.5	0.714	2
Galiza	Trabanca	Main – North Cluster	3.2	0.656	2
	Carril Garden	Main – North Cluster	3	0.617	0
	Carril Coast	Main – North Cluster	3	0.562	0
	Ribeira	Main – North Cluster	2.9	0.623	0
	Pontevedra	Main – South Cluster	3	0.563	0
	Reboreda	Main – South Cluster	3	0.628	1

Vallés, Cerdanyola del Vallés, Trabanca, Carril Garden, Ribeira, Reboreda); *Lhum-19* (Trabanca, Carril Garden, Carril Coast); and *Lihu-M1* (Trabanca). A summary of microsatellite polymorphisms is presented in Suppl. material 1: Table S2. Within the main supercolony, the Catalanian locality (Cerdanyola del Vallés) showed slightly higher levels of observed heterozygosity over all loci and a higher number of alleles (Ho = 0.714 and 3.5 alleles over all loci, Table 3) than the localities sampled in Galiza (Ho mean±SD = 0.601±0.04 and a mean of 3.0±0.1 alleles over all loci). Levels of genetic diversity (i.e., observed heterozygosity and mean number of alleles) were similar between all the localities sampled in Galiza (Table 3).

Bayesian population assignment tests including all genotyped individuals (i.e., individuals from both main and Catalanian supercolonies sampled in Catalunya plus the individuals sampled in Galiza) identified K = 2 as the value that best fits the data. Results of the analyses with STRUCTURE assigned all individuals belonging to the Catalanian supercolony to one genetic cluster, well differentiated from the cluster that includes the *L. humile* individuals from Cerdanyola del Vallés (main supercolony) and all the localities sampled in Galiza (see Fig. 7, Suppl. material 1: Fig. S2). According to these results, all populations of *L. humile* sampled in Galiza would belong to the main supercolony. Nevertheless, at values of K = 3 (and even at K = 4; see Fig. 7), there is some level of genetic differentiation within the Galizan localities, with the populations of Pontevedra and Reboreda being clearly differentiated from the rest of the samples from the region (see Suppl. material 1: Figs S3, S4). In agreement with this, the STRUCTURE analyses including only the localities belonging to the main supercolony (i.e., excluding the samples from Sant Cugat del Vallés), identified two clusters of genetically similar individuals (see Suppl. material 1: Fig. S3): the first cluster includes the Cerdanyola del Vallés population in Catalonia, along with the localities of Trabanca, Carril (both Garden and Coast) and Ribeira in Galiza (hereafter the “North” cluster); and the second includes the populations of Pontevedra and Reboreda (hereafter the “South” cluster).

In agreement with the Bayesian clustering analyses results, the highest values of genetic differentiation (F_{ST}) were found between Sant Cugat del Vallés (Catalonian

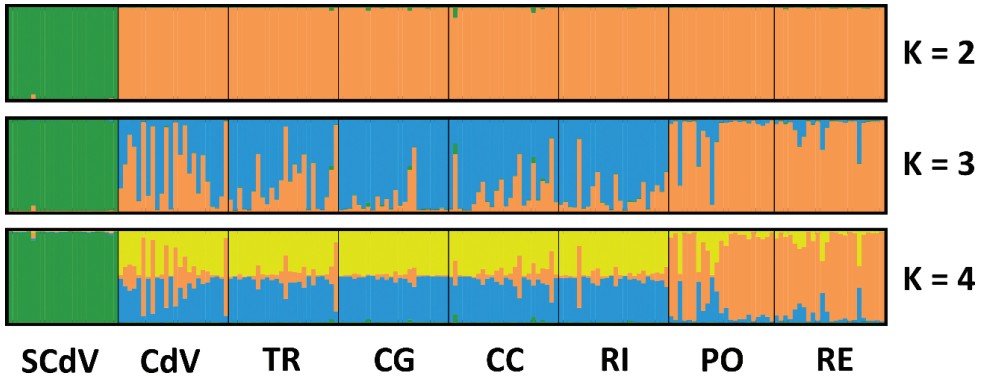


Figure 7. Genetic clustering of the eight *Linepithema humile* populations genotyped in this study, based on the seven microsatellite loci from Suppl. material 1: Table S1. Sampled localities are separated by black lines and each individual within the sampled localities is represented by a vertical bar. The proportion of colours in each bar indicates the genomic proportion derived from each genetic cluster. The plots presented here show that results of the STRUCTURE analysis with $K = 2$, $K = 3$ and $K = 4$. Populations from Galiza are listed according to the N-S geographical sampling gradient (see Fig. 1 and Table 1). Sant Cugat del Vallés: SCdV; Cerdanyola del Vallés: CdV; Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

supercolony) and the rest of the localities (i.e., main supercolony), yet pairwise F_{ST} values between the localities in the main supercolony were in most cases significant (except for the pairs Cerdanyola del Vallés – Carril Coast, Trabanca – Ribeira and Carril Coast – Carril Garden; see Table 4). These results indicate that a significant degree of differentiation exists among sampled localities, even within the same supercolony. Regarding the colonies from Galiza, F_{ST} values between each of the two genetic clusters identified by STRUCTURE ($\text{mean} \pm \text{SD}$; 0.13 ± 0.06) were significantly higher than the F_{ST} values found within each cluster (0.02 ± 0.03 ; $t = 4.59$, $df = 10.84$, $p < 0.001$; see Table 4). The levels of genetic similarity (i.e., the percent of shared alleles) were lower between the Catalanian and main supercolony (ranging from 16.7% alleles shared between Sant Cugat del Vallés and Ribeira to 25% alleles shared between Sant Cugat del Vallés and Reboreda), than between localities within the main supercolony. The percent of shared alleles within the latter was variable and ranged from 59.3% between Cerdanyola del Vallés and Redondela, to 100% between Carril Coast and Carril Garden (see Table 4). For the nests sampled in Galiza, the percent of alleles shared between nests belonging to the same genetic cluster ($\text{mean} \pm \text{SD}$; 83.03 ± 9.02) was not significantly higher than the percent of alleles shared by nests belonging to different clusters (75.67 ± 8.16 ; $t = 1.65$, $df = 12.28$, $p = 0.125$; see Table 4); but some differences in the distribution of allele frequencies could be observed between the North and the South cluster (see Suppl. material 1: Fig. S4).

For the localities sampled in Galiza, F_{ST} values were positively correlated with the geographical distance when considering either terrestrial distances ($t = 2.52$, $df = 13$, $p = 0.026$, $r = 0.57$; Fig. 5C), or the shortest distances between colonies ($t = 3.97$, $df = 13$, $p = 0.002$, $r = 0.74$).

Table 4. Population differentiation between the eight colonies of *Linepithema humile* included in this study, calculated with the data from the seven microsatellite loci listed in Suppl. material 1: Table S1. Values above diagonal represent percent of shared alleles between populations (i.e., the number of alleles shared across loci between localities/total number of alleles possessed by both localities). Values below diagonal correspond to F_{st} values. Values in bold indicate significant F_{st} values ($p < 0.05$). Negative values should be considered as zero. Sant Cugat del Vallés: SCdV; Cerdanyola del Vallés: CdV; Trabanca: TR; Carril Garden: CG; Carril Coast: CC; Ribeira: RI; Pontevedra: PO; Reboreda: RE.

	SCdV	CdV	TR	CG	CC	RI	PO	RE
SCdV		19.4	22.6	24.1	24.1	16.7	16.1	25
CdV	0.389		63	63	65.4	79.2	75	59.3
TR	0.345	0.048		76	79.2	72	76	64.3
CG	0.381	0.021	0.016		100	86	81.8	71
CC	0.398	-0.029	0.010	-0.012		86	86.4	71
RI	0.370	0.087	-0.005	0.039	0.034		85.7	69.6
PO	0.453	0.067	0.167	0.166	0.090	0.191		82.6
RE	0.383	0.114	0.121	0.171	0.125	0.131	0.077	

Discussion

Our results support those from previous studies that identified the main supercolony in the NW of the Iberian Peninsula (Giraud et al. 2002), although the results of our population genetic analyses suggest the existence of genetic divergence in Galiza and identify two genetic clusters (i.e., North and South cluster; see Fig. 1 and Suppl. material 1: Fig. S3). The genetic differences and the aggressiveness found between these two clusters was lower than that expected between supercolonies, but higher than what would be expected within the same supercolony (Holway et al. 1998; Giraud et al. 2002; Suarez et al. 2002; Tsutsui et al. 2003; Thomas et al. 2005, 2006, 2007; Pedersen et al 2006; Vogel et al. 2009; Blight et al. 2010; Van Wilgenburg et al. 2010a; Blight et al. 2012, 2017, Berville et al. 2013) as it was found in *Myrmica rubra* (Chen et al. 2018) and *Formica pressilabris* (Hakala et al. 2020).

Aggressiveness tests performed with several populations of *L. humile* from Galiza suggested potential agonism within the main supercolony (X. Espadaler, personal communication 11 February 2021; pilot test performed by us, see Material and Methods). When considering all the genotyped nests, samples from Galiza are assigned to the same genetic group as the main supercolony (Fig. 7); however, when considering the Galizan samples and the main supercolony, i.e. excluding the Catalanian supercolony, our results suggest that the samples belonging to the main supercolony from Catalunya are more similar to the Galizan North cluster than to the South cluster (Suppl. material 1: Fig. S3). In agreement with this, F_{ST} values were also higher between than within both genetic clusters identified in Galiza. F_{ST} values found between Galizan clusters (0.13 ± 0.06) were much lower than that expected between the main and the Catalanian supercolony (0.54; Jaquière et al. 2005; 0.47; Blight et al. 2012), lower than USA supercolonies (0.29 ± 0.01 ; Thomas et al. 2006), close to the values found between native supercolonies (>0.15 ; Vogel et al. 2009; between 0.095 ± 0.008 to 0.252 ± 0.007 ;

Pedersen et al. 2006), and higher than those found between the main and Corsican supercolony (0.06; Blight et al. 2012). The between and within cluster F_{ST} values found in Galiza could fit within those found between (0.015 to 0.074) and within (0.034 to 0.103) supercolonies in *Formica pressilabris* (Hakala et al. 2020). However, the genetic diversity within each of the identified clusters was similar to that observed in the main supercolony, suggesting the maintenance of genetic diversity, albeit with differences in the distribution of alleles between clusters (see Suppl. material 1: Fig. S4).

Interspecific attacks were triggered by *L. humile* independently of the nest and season and these attacks were performed faster than the intraspecific attacks performed towards conspecifics from different nest. This suggests that ants were able to correctly identify their conspecifics but the inter-individual differences were sufficient to cause agonistic responses. We consider that the previous intraspecific aggressions observed in *L. humile* in the introduced areas (Tsutsui et al. 2000; Roulston et al. 2003) were due to the analysis of different supercolonies based on posterior studies that found other supercolonies in the studied areas (e.g. Buczkowski et al. 2004; Thomas et al. 2005). Therefore, we show for the first time the existence of aggressiveness within the same supercolony in *L. humile*. Although we did not find a clear pattern showing higher aggressiveness between the identified genetic clusters than within them, we found trends supporting this fact. For instance, up to 50% of attacks were registered between clusters and only up to 15% were registered within clusters. Furthermore, mortality was only found between clusters in spring while in autumn this was mainly found between clusters (up to 35% of cases) and less within them (<7.5%).

We found a significant correlation between genetical and geographical distances, with more distanced colonies being the most genetically different (Fig. 5C). This supports the definition of supercolonies proposed by Pedersen et al. (2006) in which they assumed a lack of individual flow between distanced nests of the same supercolony, and previous studies performed in California (Thomas et al. 2007). Higher relatedness between closer nests of the supercolony was proposed as sufficient to maintain kin-selection in *L. humile* although when local relatedness trends to zero (but see Vogel et al. 2009; Helanterä 2022). However, differences in aggressiveness could not be explained either by the geographical distances or the genetic dissimilarities between colonies (Fig. 5A, B). Similarly, no correlation between aggressiveness and geographical or genetic distances was found in other supercolonies (Giraud et al. 2002; Thomas et al. 2006, 2007; Vogel et al. 2009; but see Hakala et al. 2020).

All nests sampled in Galiza showed a higher expression of behaviors associated with invasiveness in autumn than in spring, except for the number of foragers, which was higher in spring. Seasonality determined workers' behavioural pattern: individuals were more proactive in autumn (more explorer, less thigmotactic, and more aggressive) than in spring. However, the number of foragers was higher in spring than in autumn. Foraging (forager abundance and recruitment) is highly dependent on the species, but also on temperature and habitat (Stuble et al. 2013). Our results could be interpreted according to differences in decision-making, which depends on the individual condition (Brodersen et al. 2008) and personality (Gambetti and Giusberti 2019). Proactive ants (emerged in

summer-autumn) facing resources could focus on carrying the resource because of their higher efficiency to do it, while reactive ants (emerged in winter-spring) due to their behavioural limitations, could focus on the group force or the recruitment of other workers.

As has been described in *Paratrechina flavipes* (Ichinose 1991), *L. humile* showed higher aggressiveness in autumn, when ants express higher activity levels. A similar trend was also found in *L. humile* introduced in the USA (Thomas et al. 2006, 2007). This suggests that workers' behaviour is conditioned by their biological cycle and/or the polydomy dynamics of the species. Lower temperatures diminish *L. humile* workers' activity and queens' fertility (Benois et al. 1973; Abril et al. 2018). Nests that remain separated in summer aggregate into hibernation nests, probably favoured by the activity slowdown in workers and queens (showed in California, Markin 1970; France; Benois et al. 1973; Argentina, Heller and Gordon 2006), the seasonal cuticular change (Abril et al. 2018), and the higher cost involved in defending a territory against multiple neighbour colonies (Giraud et al. 2002). On the other hand, the benefits in survivorship and fertility produced by aggregation (Luque et al. 2013) should also promote winter aggregations. In our experiment, ants fought similarly against *M. rubra* in both seasons, suggesting that seasonal differences in the intraspecific aggressiveness were not due to a reduction in the species agonistic behaviour but a mechanism mediated by the life history of the species. The seasonal effect could explain why we found aggressiveness in Galiza (only in autumn but not in spring) while previous studies performed over the same region carried out in spring (Giraud et al. 2002) did not find the same agonisms. However, aggressiveness tests were performed in other studies at different seasons and never found within supercolonies' agonism (native range: May (Blight et al. 2017), October–November (Vogel et al. 2009), December (Suarez et al. 1999); USA: April to September (Thomas et al. 2007), mainly in spring and summer (P. Nonacs, pers. comm. 2020), August, adding samples collected in the field every month for one year (Suarez et al. 2002), October (Thomas et al. 2006), November to March (Suarez et al. 1999); mainland Europe and islands: May (Blight et al. 2017), June (Wetterer and Wetterer 2006; Blight et al. 2010), October (Blight et al. 2012)). Future studies should decipher the underlying mechanism that drives the seasonal dependence of aggressiveness between colonies within the same supercolony.

We consider that our main results (low genetic differences, low aggressiveness within supercolony nests) support a better fit with the hypothesis of an evolutionary process of divergence in *Linepithema humile* linked to the development of agonistic interactions within the main supercolony rather than with the hypothesis of multiple introductions of native colonies. Aggressiveness within the same supercolony could be explained by differences in cuticular compounds caused by experienced local environmental factors as the diet (in *L. humile*, Liang and Silverman 2000; Buczkowski et al. 2005; but see Giraud et al. 2002), nest isolation (in *Lasius flavus*, Pontin 1961), season (in *Paratrechina flavipes*, Ichinose 1991) and/or genetic variability (Thomas et al. 2006; this study). Aggressiveness could act as a behavioural barrier limiting individual flow between nests and thus, increasing genetic differences between them. Evolutionary divergence was already suggested to explain the hypothetical emergence of the Corsican

supercolony from the main supercolony (Helanterä 2022), which begs the question as to whether the other European supercolony detected - the Catalanian supercolony - is also a product of an older divergence or an introduction of a second supercolony.

It must be taken into account that we have considered only two possible explanations for the existence of competition between *L. humile* nests located in the same region (new introductions and evolutionary divergence) due to the lack of within supercolony aggressiveness reported in previous studies and the rarity of new supercolonies founded by flying queens (Helanterä 2022). However, within supercolony competition could be produced without any of the cited mechanisms, and be more common than assumed (see Hakala et al. 2020). It was also shown that unicolonial species can be organized in aggressive supercolonies (Vogel et al. 2009); supercolonies could be constituted by a “fluid mosaic of aggressive and amicable interactions” (Hakala et al. 2020) between connected nests genetically and behaviourally differentiated (clusters or “subcolonies” according to Helanterä 2022). This could limit the functional unity of the introduced supercolonies (see Gordon 2010) and the connectivity of distanced nests (Pedersen et al. 2006). We could argue that the genetic and behavioural differences found could be also due to an ancient merging of supercolonies as assumed in *Lepisiota canescens* (Sorger and al. 2017) and *Formica paralugubris* (Holzer et al. 2009). However, this phenomenon seems unlikely due to the high aggressiveness and low individual flow between supercolonies of *L. humile*, as well as the relatively recent introduction of the species, which limits the time for colony merging. Another hypothesis not explored here is that the variability observed in Galiza is due to the introduction of a supercolony that was previously introduced in another region. Although we cannot discard this idea, we consider that the probability of introducing a different supercolony from the main supercolony is low due to the presence of this supercolony around the world (Van Wilgenburg et al. 2010b; Blight et al. 2012) and if this was the case, we would expect to find higher genetic variability than the one we found. Future studies should verify the possible origin of the genetic clusters found from other introduced supercolonies. In any case, we believe that our findings provide essential preliminary conditions for studies focusing on the future invasiveness of *L. humile* and those focused on the control of this species.

To conclude, our results point to divergent evolution as a possible cause of the incipient genetic divergence and behavioural variability found in the NW Iberian Peninsula. In addition, we showed a strong seasonal effect that conditions the expansion (exploration, use of open areas), efficiency (foraging), and aggressiveness of the nests of the sampled locations, suggesting competition within the supercolony. Considering the lack of competition within supercolonies as the main force of invasion for this species, our results showing agonism between nests of the same supercolony signal a weak point for this introduced species. In line with previous results, our study contributes to the development of conservation and management plans to control this species and to prevent the colonisation of new habitats. Conservation plans should be designed taking into account the season and the homogeneity of the nests, considering higher invasive potential for nests sharing similar traits and higher plasticity for those showing variability (Sanmartín-Villar

et al. 2021). Our findings could also contribute to a better understanding of the eco-ethology of the supercolony phenomenon and its evolutionary processes. Future studies should focus on understanding the origin of within supercolony variability.

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Supplementary material 1

Figures S1–S4, Tables S1, S2

Authors: Iago Sanmartín-Villar, Everton Cruz da Silva, Violette Chiara, Adolfo Cordero-Rivera, M. Olalla Lorenzo-Carballa

Data type: Docx file.

Explanation note: Genetic divergence and aggressiveness within a supercolony of the invasive ant *Linepithema humile*.

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Supplementary material 2

Behavioural data

Authors: Iago Sanmartín-Villar, Everton Cruz da Silva, Violette Chiara, Adolfo Cordero-Rivera, M. Olalla Lorenzo-Carballa

Data type: Data.

Explanation note: Data obtained from the behavioural tests.

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Recurring infection by crayfish plague pathogen only marginally affects survival and growth of marbled crayfish

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Abstract

Invasive alien crayfish threaten the diversity of freshwater ecosystems and native crayfish fauna. In Europe, this is largely due to transmission of the crayfish plague to susceptible native crayfish. Many invasive species tolerate crayfish plague, but the infection still has the potential to reduce the fitness of a tolerant host due to energy trade-offs between immune response maintenance and life-history traits, such as growth and reproduction. In combination with other unfavourable conditions, such a response could alter further invasion success of an otherwise successful crayfish invader. We examined whether repeated infection with one of the most virulent haplogroups of crayfish plague agent (*Aphanomyces astaci*) affects growth or survival of the juvenile marbled crayfish (*Procambarus virginalis*). Juveniles were exposed to i) two levels of pathogen concentrations, and ii) two different feeding regimes under the higher pathogen concentration. In all performed trials, repeated infection reduced growth rates, while the combination of recurring infection and food limitation significantly increased mortality. The average energy cost of the immune response was estimated at 12.07 J/day for individuals weighing 0.3 grams. Since infections were frequent

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and pathogen concentrations high, results suggest that marbled crayfish is resistant to *A. astaci* pathogen and its survival is only affected by adding the stress of food limitation. The survival of almost half of the individuals exposed to high pathogen loads and extreme food limitation indicates that chronic infection by crayfish plague is unlikely to be an important factor impeding invasion success of the marbled crayfish, even under harsh conditions. Our results add to the growing body of evidence that marbled crayfish has potential to become one of the most successful freshwater invaders.

Keywords

food limitation, freshwater, immunity cost, infection, invasive species, trade-off

Introduction

Invasive alien species drive biodiversity loss and impair ecosystem services worldwide (Pyšek and Richardson 2010), partly because they carry pathogens to which native species lack resistance. In freshwater ecosystems, crayfish directly affect ecosystem structure and diversity (Dorn and Wojdak 2004). Many native crayfish, particularly in Europe, are on the brink of extinction due to introductions of invasive crayfish and their pathogens (Jussila et al. 2021; Theissinger et al. 2021). Invasive crayfish imported from North America into Europe introduced a novel and lethal disease, the crayfish plague, caused by the oomycete *Aphanomyces astaci* Schikora, 1906 (Jussila et al. 2021; Theissinger et al. 2021). The introduction and transmission of pathogen *A. astaci* is the main reason why native crayfish were displaced by invaders, and crayfish plague is solely responsible for decimation of numerous native European crayfish populations (Martín-Torrijos et al. 2019; Jussila et al. 2021; Theissinger et al. 2021).

North American crayfish species tolerate the crayfish plague due to their rapid immune reaction to the pathogen. The fast activation of the prophenoloxidase (ProPO) system encapsulates the pathogen's hyphae in melanin (Cerenius et al. 2003). However, even in North American crayfish, stressful conditions (i.e. chronic infections, unfavourable environmental conditions) may increase their susceptibility to pathogens (Chinchio et al. 2020) or negatively affect their fitness, growth and development (cf. Francesconi et al. 2021). Chronic infection requires continuous mounting of an immune response even in a tolerant species, thus incurring energy costs. In other species, covering these costs requires energy redistribution that results in trade-offs with life-history traits, such as growth and reproduction (e.g. Lochmiller and Deerenberg 2000; Martin et al. 2003, 2008; Rantala and Roff 2005; Lee 2006; van Der Most et al. 2011; Brown and Shine 2014; Schwenke et al. 2016; Kirschman et al. 2017; Körner et al. 2017; Miyashita et al. 2019). These trade-offs are most apparent under stressful conditions (i.e. limited resources or environmental/physiological stress; Kirschman et al. 2017; Rumschlag and Boone 2020) and may cause death (Garner et al. 2009) and ultimately adversely affect host population dynamics (Boots and Norman 2000; Boots et al. 2003; Campbell et al. 2019). Therefore, while chronic exposure to *A. astaci* may be sublethal in a resistant invasive crayfish species, it can cause mortality if individuals are under additional stress (Aydin et al. 2014). Such circumstances may lead to

population crashes even in tolerant invaders (e.g. as in signal crayfish; Edsman et al. 2015), alter their further invasion success, and even mitigate their negative effects on the ecosystem (Fincham et al. 2019; Chinchio et al. 2020).

We analyse whether chronic exposure to *A. astaci* may lead to significant sublethal or lethal effects in the marbled crayfish (*Procambarus virginalis* Lyko 2017, Cambaridae), a high-risk crayfish invader in Europe (Chucholl 2016; Kouba et al. 2021; Vogt 2021), listed amongst the Invasive Alien Species of Union concern (EU Regulation No. 1143/2014). Marbled crayfish is the only obligatory parthenogenetic freshwater crayfish (Scholtz et al. 2003) producing a large number of genetically identical offspring (Martin et al. 2007; Vogt et al. 2008), which makes it an ideal model organism. Although genealogically most closely related to North American species *Procambarus fallax* (Hagen, 1870), it has no known native habitat (Lyko 2017; Hossain et al. 2018). Its occurrence in Europe is significantly increasing due to its popularity in pet-trade (Chucholl 2016), as are contacts with other crayfish invaders that are known *A. astaci* carriers (cf. Linzmaier et al. 2020). Marbled crayfish can carry *A. astaci* haplogroup D, associated with another invader, *Procambarus clarkii* (Girard, 1852) (Keller et al. 2014; Mrugała et al. 2015). It is resistant to low concentrations of a highly virulent *A. astaci* haplogroup B, associated with invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852) (Francesconi et al. 2021). However, the impact of chronic infection by crayfish plague and its combined effect with other stressors on survival and life history of marbled crayfish have not been explored yet, even though they could potentially affect its future expansion.

Methods

Experimental animals

Juvenile marbled crayfish were taken from an adult female transferred from Šoderica Lake in northern Croatia (46°14'20.9"N, 16°54'33.6"E) to the laboratory of the Department of Biology at the Faculty of Science in Zagreb. Adult marbled crayfish were kept in the laboratory for five months prior to oviposition, when juveniles for the experiments were obtained. For marbled crayfish keeping and research, we obtained a licence from the responsible Ministry of Environment and Energy, Croatia (Licence number: UP/I-612-07/19-43/01; 517-05-1-1-20-6), in accordance with the EU Regulation on Invasive Alien Species (EU Regulation No. 1143/2014).

Juvenile marbled crayfish were used in all experiments, since they moult often, allowing faster assessment of effects of exposure to *A. astaci* pathogen on growth and since juvenile growth is linear (McLay and van den Brink 2016).

Housing of experimental animals

Juvenile marbled crayfish were kept individually in 100 ml of aerated tap water in 250 ml plastic containers with a diameter of 90 mm. Containers were placed in cooled

incubators (ST 6 COME, POL-EKO-APARATURA, Poland) 16–18 days before the start of the experiments to allow the juveniles to adapt to housing conditions (temperature regime 20 °C, photoperiod 12:12 light:dark). To avoid cross-contamination, each experimental group (described below) was housed in a separate incubator. The selected temperature results in the lowest mortality in the laboratory (Vogt et al. 2004). An aquarium pump (JK-AP9500, JK ANIMALS, Czech Republic) was placed between the containers and all containers were covered with a plastic lid to prevent water evaporation and escape of the crayfish. The crayfish were fed one pellet of JBL NovoPrawn food for shrimps with frequency depending on the feeding regime, and the water in all the containers was changed twice a week.

Aphanomyces astaci zoospore production

Haplogroup B strain of *A. astaci*, associated with the signal crayfish (Francesconi et al. 2021), was selected for the experiment because of its potential horizontal transmission from the signal crayfish to marbled crayfish. Šoderica Lake is close (ca. 450 m) to the Drava River, which contains signal crayfish infected with *A. astaci* (Maguire et al. 2016) and contact between the two species is expected to occur (Dobrović et al. 2021). Additionally, the signal crayfish is the most widespread invasive crayfish species in Europe, present in over 29 EU countries and in EU candidate countries (Dragičević et al. 2020), making transmission of this highly virulent haplogroup to the marbled crayfish likely.

A. astaci PEC8 isolate (haplogroup B) was obtained from Prof. Frédéric Grandjean from University of Poitiers, France. The isolate was grown on PG1 agar (Unestam 1965) with addition of antibiotics - oxolinic acid (SIGMA 0-00877) and ampicillin (SIGMA A 9518) at a concentration of 10 mg/l, to prevent bacterial growth. *A. astaci* zoospore production was induced using a protocol, based on Makkonen et al. (2012). A piece of PG1 agar (4 × 4 mm) containing *A. astaci* hyphae was used to inoculate 45 ml of liquid PG1 medium (Unestam 1965) with antibiotics in 12 replicates. The replicates were incubated at 18 °C for four days, after which the hyphae were cut into small fragments and agar was removed. Total hyphae biomass was divided into four portions, each portion was transferred into 50 ml of fresh liquid PG1 medium with antibiotics and grown at 18 °C for three days. Liquid PG1 medium was then removed and sporulation was induced by washing the hyphae in water from the stream Vrapčak in Gornje Vrapče, Zagreb (45°50'36.3"N, 15°53'44.8"E). Hyphae were washed four times with 200 ml of autoclaved natural water with mild linear shaking for 45 minutes at 18 °C, and were left in the shaker for 20 hours in the last (fourth) wash. Hyphae were then removed and zoospore concentration in the suspension was counted using a Thoma counting chamber. Crayfish infection was performed immediately after the zoospore production by adding zoospores to crayfish containers. Experimental zoospore concentrations (described below) in 100 ml of water were obtained by diluting the produced zoospore suspension with aerated tap water.

Experimental design

Prior to the infection experiment, virulence of the PEC8 isolate and sublethal load was tested in a pilot experiment to determine two sublethal concentrations of *A. astaci* zoospores and frequency of the repeated infections (Vukelić 2021). Infections were set to be repeated every two weeks at concentrations of 7500 zoospores/ml and 15000 zoospores/ml in Experiment 1, and 15000 zoospores/ml in Experiment 2 (described below). Dosages were higher than in other studies involving infection trials on marbled crayfish (i.e. Francesconi et al. 2021), but still within a range used for tolerant crayfish invaders (i.e. 10000 zoospores/ml in signal crayfish infection trials; Aydin et al. 2014) and considered realistic in the case of acute infection in the wild (Aydin et al. 2014).

During the experiments, crayfish from all experimental groups (control and infected groups) were photographed and weighed i) directly before each infection, ii) two weeks after the last infection (Experiment 1 and 2), and iii) six weeks after the last infection (Experiment 1). Due to the small size of the juveniles, their total length (TL; from the top of the rostrum to the end of the telson, in mm) was measured from photographs using the image processing programme, ImageJ (Schneider et al. 2012; <http://rsbweb.nih.gov/ij/>). Each crayfish was measured three times and the mean value represented TL. Before weighing, each crayfish was carefully dried on filter paper to remove excess water and weighed using a digital scale (accuracy: ± 0.0001 g). During the experiment, we also recorded mortality, disease symptoms (melanisation of the cuticle) and moulting of the crayfish. At the end of the experiments, surviving crayfish were euthanised by a rapid cut of the nerve cord through the thorax to the abdomen, in accordance with available guidelines for humane killing of crayfish (Conte et al. 2021). All specimens were stored in 96% ethanol for detection of *A. astaci* DNA from crayfish cuticle as described in Suppl. material 1: S1, while in surviving crayfish from Experiment 2, hepatopancreas was additionally dissected for gene expression analysis (described below).

Experiment 1: Repeated exposure of juvenile marbled crayfish to different *A. astaci* zoospore concentrations

A total of 55 juveniles were used in this experiment. For the trial, juvenile marbled crayfish were randomly divided into three experimental groups: 1) control group (15 individuals, non-infected), 2) group 7500 (20 individuals infected with 7500 zoospores/ml), and 3) group 15000 (20 individuals infected with 15000 zoospores/ml). The infection experiment was conducted for 18 weeks, with *A. astaci* infection performed every other week, six infections in total. In one case, the third infection was performed four weeks after the second infection due to unsuccessful *A. astaci* zoospore production at the time. Surviving crayfish were euthanised six weeks after the last infection and the cuticle of all crayfish was tested for presence of *A. astaci* (those that died during the experiment and those that survived until the end of the experiment; crayfish plague detection described in Suppl. material 1: S1).

Experiment 2: Repeated exposure of juvenile marbled crayfish to *A. astaci* under differing feeding regimes

A total of 60 individuals from a separate batch were used to test the interaction of chronic exposure to *A. astaci* with food availability. While food availability is unlikely to be a limiting factor in nature because crayfish are omnivores (Holdich 2002), it was used as a proxy for density-dependent effects. Juvenile crayfish form social dominance hierarchies (Issa et al. 1999; Sato and Nagayama 2012) and dominant individuals have increased access to food (Herberholz et al. 2007). Increasing population density is thus expected to limit resource availability, increase competition intensity (Capelli and Hamilton 1984), and induce stress.

In this experiment, crayfish were adapted to laboratory conditions for 16 days during which all individuals were fed twice a week. Then, four experimental groups of 15 individuals were used: 1) control - fed five times a week, non-infected, 2) control, food-restricted - fed once a week, non-infected, 3) infected - fed five times a week, and 4) infected, food-restricted - fed once a week. This experiment was conducted for 12 weeks, also with *A. astaci* infection intervals every other week (five in total).

Surviving crayfish were euthanised two weeks after the last infection and cuticle samples of all crayfish were tested for *A. astaci*, as in Experiment 1 (crayfish plague detection described in Suppl. material 1: S1). Additionally, euthanised crayfish were dissected and the hepatopancreas from each was carefully removed and stored in an RNA stabilising agent (RNA later; Sigma Aldrich, MO, USA) at -80 °C until RNA extraction for gene expression analysis. Five random individuals from each group were analysed for changes in expression of two genes related to crayfish innate immunity (prophenoloxidase - ProPO and CCAAT/enhancer-binding protein beta - C/EBP- β), and central metabolic pathways of glycolysis and citrate cycle (citrate synthase - CS and glyceraldehyde 3-phosphate dehydrogenase - GAPDH), selected based on Boštjančić et al. (2021) and Zheng et al. (2021). Total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA quality was checked on the NanoVue Spectrophotometer, and RNA quantity with the QuantiFluor RNA System on the Quantus platform (Promega, USA). For the cDNA synthesis, 1 μ g of total RNA was reversely transcribed with the iScript Select cDNA Synthesis Kit (Bio-Rad, USA) and Oligo(dT)15 primer. For the quantitative reverse transcription PCR (RT-qPCR), target loci were amplified in 10 μ l reactions with iTaq Universal SYBR Green Supermix (Bio-Rad, USA), with 1 μ l of input cDNA template on the CFX Opus 96 Real-Time PCR (Bio-Rad, USA). All samples were run in duplicates with the standard deviation of cycle threshold values < 0.5. Primer pairs for all target genes and endogenous control (elongation factor 1- α) can be found in Suppl. material 1: Table S2.1. The difference in the gene expression values between the target and control samples was expressed according to the delta-delta Ct method ($2^{-\Delta\Delta Ct}$; Livak and Schmittgen 2001).

Estimating energy cost of the infection

We estimated energy cost of immunity response to chronic infection based on the differences in weight of non-infected and infected food-restricted groups in Experiment

2. Neither of the food-restricted groups grew appreciably in length, so differences in weight change between the two groups stem from differences in energy reserve dynamic: if size is the same, lighter individuals have smaller energy reserves. The reduction in energy reserves can be explained either by smaller energy input (e.g. reduction in food intake due to infection) or a higher basal metabolic rate related to the infection. Food intake was equal for both groups because all food had been eaten, so reduction in energy reserves must have been caused by increased metabolism due to the infection. Therefore, the energy content corresponding to the difference in weight represents the cost of the infection. In crayfish, changes in energy reserves are primarily reflected in change of hepatosomatic index, i.e. hepatopancreas weight (Jussila and Mannonen 1997; Sacristán et al. 2017). Energy content of hepatopancreas (E; kJ/g) of marbled crayfish was estimated using the correlation with moisture content of hepatopancreas (M; %) as suggested by Jussila and Mannonen (1997), using the regression for *Astacus astacus* (Linnaeus, 1758), for pooled sexes:

$$E = -0.336M + 54.189. \quad (1)$$

Average moisture content of hepatopancreas of marbled crayfish from our sampling site was reported to be 62% (Žižák 2015) and, therefore, this value was used to calculate energy reserve content. Finally, the difference in weight change between the groups was multiplied by the estimated energy content and divided by the duration of experiment to obtain cost of immunity response per day.

Statistical analysis

The effects of repeated infections using two *A. astaci* concentrations and interactive effects of repeated infections and food availability were examined through three major endpoints: 1) total growth (i.e. weight/length gain throughout the total duration of both experiments), 2) rate of growth (i.e. weight/length increment over time, in each experimental week, presented in Suppl. material 1: S3–S5), and 3) mortality (measured through the whole experimental period). In addition, the changes in immune and metabolic gene expression and metabolism were analysed in Experiment 2. Statistical analyses and graphical representation of results were performed in the R statistical environment (R version 3.6.3, R Studio 1.2.5033). The ‘base’ and ‘pastecs’ packages (Grosjean and Ibanez 2018; R Core Team 2020) were used for descriptive statistics and basic homoscedasticity (Levene test), normality (Shapiro-Wilks, QQ-plot) and extreme value tests, while the ‘ggplot2’ package was used for graphical display.

Total growth

Robust ANOVAs based on trimmed means (20% of trimming level; Wilcox 2012; Mair and Wilcox 2020; ‘WRS2’ package in R) were used to test (i) the effects of *A. astaci* infection (Experiment 1; one-way factorial design; three levels), and (ii) the joint effects of *A. astaci* infection and food availability (Experiment 2; two-way

factorial design; 2×2 levels) on total weight (length) gain. Total weight (length) gain was calculated as the difference between the final weight (length) at the end of the experiment and the initial weight (length) at the beginning of the experiment. Robust ANOVAs were used instead of classical ANOVAs to overcome problems associated with deviations from homoscedasticity and to reduce the influence of outliers observed in the data. Post-hoc tests were also performed in the robust ‘*WRS2*’ environment (Mair and Wilcox 2020), while p-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) method. The ‘*multcompView*’ package (Graves et al. 2019) was used to convert the vector of p-values to a character-based display in which common characters denote levels or groups that are not significantly different.

Rate of growth

Growth rate analysis was performed to analyse questions such as: (a) what average growth trajectory best describes the rate of growth over time for all crayfish, (b) what is the variability in growth rates across crayfish, and finally, (c) does *A. astaci* infection and food availability explain variability in growth rates? Growth rate analysis, unlike total growth analysis, is based on complete longitudinal measurements of weight (and/or length) rather than just initial and final values. As the measurements at specific time points are clustered (nested) within individual crayfish, they imply a hierarchical structure of the data and intra-individual correlation. Therefore, the multilevel modelling (MLM) technique was used for data analysis (Peugh 2010; Monsalves et al. 2020). A detailed specification of all data levels, model variables and model equations can be found in Suppl. material 1: S3.

Mortality

Fisher’s exact tests were used to determine the association between exposure to *A. astaci* and mortality in both experiments. Pairwise (post-hoc) Fisher test from the ‘*rstatix*’ package in R (Kassambara 2020) with the Holm method of adjusting p-values was used for multiple comparisons of groups in Experiment 2. Kaplan-Meier estimates (curves) of survival probability and comparison of survival times between groups by the log-rank test were performed using the ‘*survival*’ and ‘*survminer*’ packages in R (Kassambara et al. 2020; Therneau 2022).

Gene expression

The Kruskal-Wallis test was used to determine whether the expression of immune and metabolic genes differed significantly between the groups in Experiment 2. Dunn’s test from the ‘*FSA*’ package in R (Ogle et al. 2021) was used for multiple pairwise comparisons of the groups. In the Dunn test, p-values were adjusted according to

the Holm method. To further investigate the relationships between gene expression variables, the ‘*FactoMineR*’ and ‘*factoextra*’ packages in R (Le et al. 2008; Kassambara and Mundt 2020) were used for principal component analysis (PCA) of the gene expression dataset.

Data availability

Data are permanently deposited in an open repository (Dryad: <https://doi.org/10.5061/dryad.3xsj3txkp>).

Results

Infection success

In both experiments, all crayfish that died (marked in red colour in Fig. 1) tested *A. astaci* positive, while all control animals tested to be pathogen-free (Fig. 1). Overall, the proportion of *A. astaci* positive individuals was higher in Experiment 2 (*A. astaci* detection two weeks since the last infection; Fig. 1B) than in Experiment 1 (*A. astaci* detection six weeks since the last infection; Fig. 1A).

Experiment 1: Repeated exposure of juvenile marbled crayfish to different *A. astaci* zoospore concentrations

Total growth

Repeated exposure to the crayfish plague pathogen (Fig. 2A) significantly reduced total weight gain ($F(2,17.06) = 10.04$, $p = 0.001$, effect size $\xi = 0.84$, $CI(\xi) = [0.64, 1.11]$; Suppl. material 1: Table S4.1C). Both exposed groups weighed less than the control group, but did not differ from each other (Fig. 2A; post-hoc tests in Suppl. material 1: Table S4.1E). Total length gain (Suppl. material 1: Fig. S4.1A) was not significantly different between treatments ($F(2,17.19) = 1.86$, $p = 0.185$; Suppl. material 1: Table S4.1D).

Rate of growth

Due to the complexity of the MLM model, a detailed description of the results on individual and group growth rates is given in Suppl. material 1: S3.2, S4, Figs S4.2, S4.3, Tables S4.2, S4.3). Here, we provide only a summary of the main results (fixed effects). *A. astaci* infection significantly reduced growth rates: the growth rate of weight in the control group (12.7 mg/wk) was significantly higher

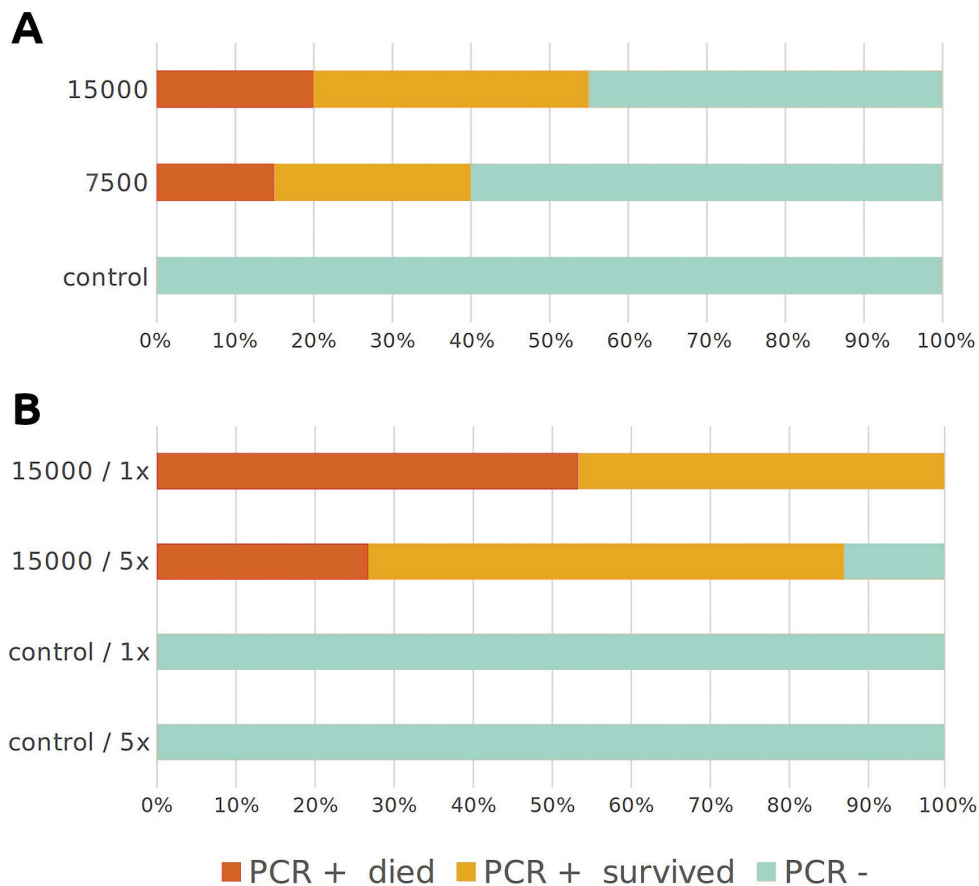


Figure 1. Detection of *A. astaci* in the cuticle of marbled crayfish in **A** Experiment 1 and **B** Experiment 2. In Experiment 1, surviving crayfish were euthanised six weeks after the last infection; in Experiment 2, they were euthanised after two weeks. PCR+ = *A. astaci* detected, PCR - = *A. astaci* not detected.

than the growth rates in both *A. astaci* infected groups (10.2 mg/wk and 9.6mg/wk; Suppl. material 1: Table S4.2D), while growth rate of length was significantly reduced only under higher *A. astaci* concentration (Suppl. material 1: Table S4.3D). Weight and length increased significantly over time in all groups.

Mortality

The effect of exposure to pathogens on mortality was not significant ($p = 0.26$, Fisher's exact test). A similar result, i.e. insignificant effect of infection, was also inferred from the overlapping confidence intervals of the Kaplan-Meier survival curves (Fig. 2C) and from the comparison of survival times between the groups using the log-rank test ($\chi^2(2) = 3.2$, $p = 0.20$).

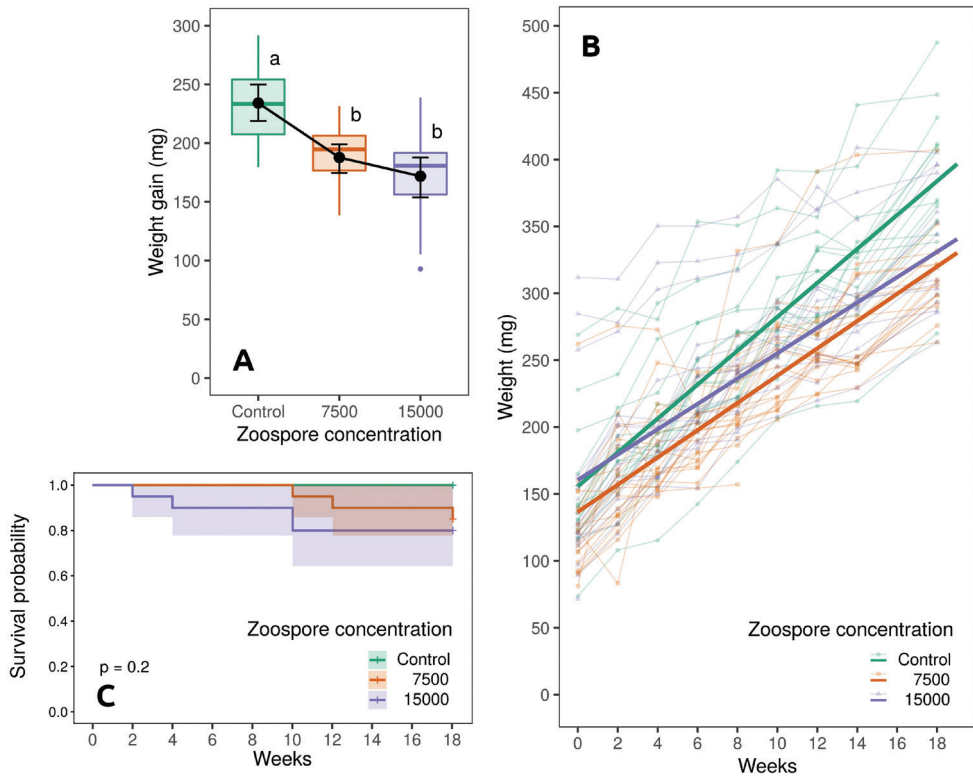


Figure 2. Effects of repeated infection of marbled crayfish juveniles using two *A. astaci* zoospore concentrations (7500 and 15000 zoospore/ml) on **A** total weight gain (total growth) **B** rate of weight increment (rate of growth) and **C** survival probability of individuals. Different letters in panel A denote significant differences, error bars represent 95% confidence intervals (CIs) around the mean. Results for total length gain and rates of length increment are presented in Suppl. material 1: S4.

Experiment 2: Repeated exposure of juvenile marbled crayfish to *A. astaci* under differing feeding regimes

Total growth

Food availability (i.e. different feeding regimes) significantly influenced crayfish growth (Fig. 3A). There was a significant effect of food ($Q = 652.22$, $p = 0.001$), *A. astaci* infection ($Q = 44.55$, $p = 0.001$), and their interaction ($Q = 11.92$, $p = 0.003$) on the total weight gain during the experiment (Suppl. material 1: Table S5.1C). Pairwise post-hoc comparisons revealed significant differences in weight gain between all groups (Suppl. material 1: Table S5.1D). Food-restricted groups gained significantly less weight - both in the control ($\hat{\psi} = 279.11$, $p < 10^{-5}$) and the infected groups ($\hat{\psi} = 212.62$, $p < 10^{-5}$). Weight gain of the control food-restricted group was approximately 90% lower than in the control group fed five times

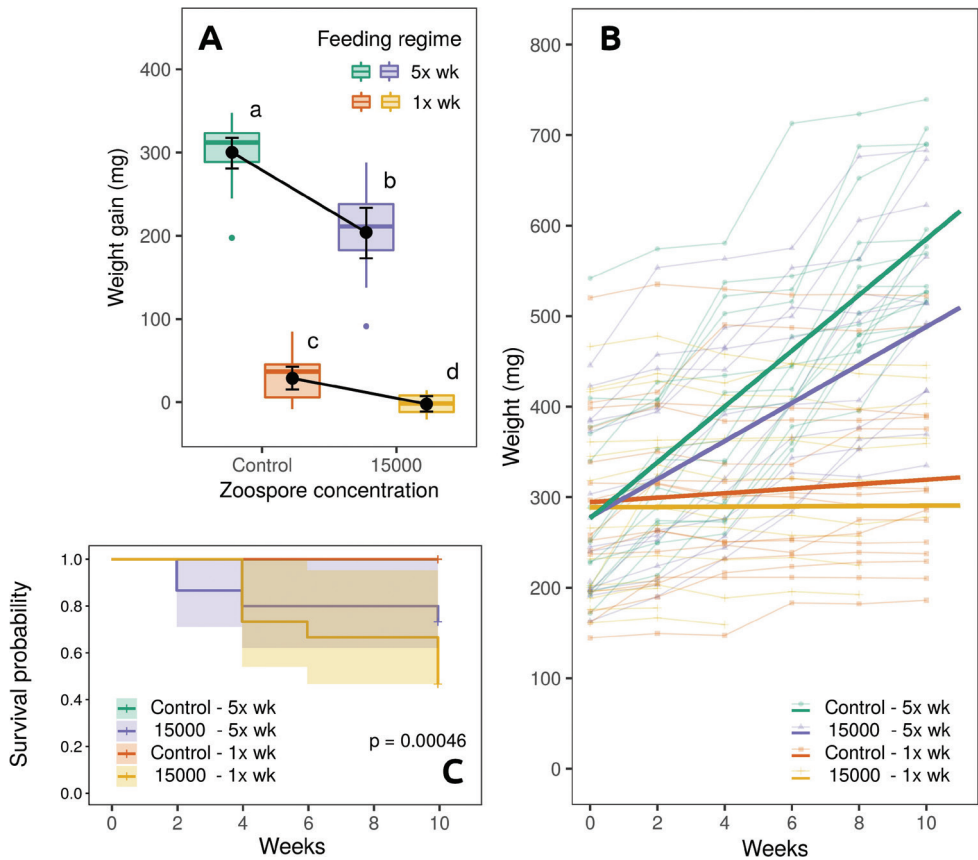


Figure 3. Effects of repeated infection of marbled crayfish juveniles with 15000 zoospore/ml of *A. astaci* under two feeding regimes (once a week and five times a week) on **A** total weight gain (total growth) **B** rate of weight increment (rate of growth) and **C** survival probability of individuals. Different letters in panel A denote significant differences, error bars represent 95% confidence intervals (CIs) around the mean. Results for total length gain and rates of length increment are presented in Suppl. material 1: S5.

a week (Suppl. material 1: Table S5.1A). Total weight gain was lower when crayfish were infected, compared to non-infected control groups (feeding regime five times per week: $\hat{\psi} = 97.50$, $p = 0.0002$; feeding regime once a week: $\hat{\psi} = 31.01$, $p = 0.014$; Suppl. material 1: Table S5.1D). In general, the effect of food on weight gain ($\xi = 0.93$, $CI(\xi) = [0.89; 1]$) was stronger than the effect of *A. astaci* infection ($\xi = 0.21$, $CI(\xi) = [0.03; 0.56]$). Furthermore, the effect of infection was stronger at high food than food-restricted regime due to significant interactions between food and weight (Suppl. material 1: Table S5.1C). Additional inferential analysis of length as a response variable leads to similar conclusions, but without significant differences in total length gain between infected and non-infected groups under food restriction ($p = 0.07$; Suppl. material 1: Fig. S5.1A, Table S5.1B, E, F).

Rate of growth

A detailed analysis of the results of the MLM model can be found in Suppl. material S3.3 and S5, Figs S5.2, S5.3, Tables S5.2, S5.3); here, a summary of the main results (fixed effects) is given. Both food restriction and infection with *A. astaci*, as well as their interactive effect, significantly reduced growth rate of weight (and length). The average growth rate of uninfected crayfish fed five times per week was 30.8 mg/wk, compared with an average growth rate of 21.0 mg/wk for the infected group under the same feeding regime, and an average growth rate of 2.5 mg/wk for the food-restricted control group. The average growth rate of the infected crayfish with food restriction was only 0.2 mg/wk. The infection-induced decrease in growth rate was greater when food was abundant than when food was restricted.

Mortality

Food limitation and *A. astaci* infection increased individual mortality. Mortality differed significantly amongst groups in Experiment 2 ($p < 10^{-3}$, Fisher's exact test), with the highest mortality occurring in the infected group fed once a week (8 deaths, i.e. 53% in 10 weeks; Fig. 3C). The statistically significant difference in mortality in pairwise comparisons was obtained only between this group and (i) the control (non-infected) group fed once per week ($p = 0.013$) and (ii) the control group fed five times per week ($p = 0.013$). The difference in mortality rate between the infected and non-infected groups fed five times a week was not significant ($p = 0.398$). Consistent with the result of Fisher's test, survival times estimated by Kaplan-Meier method (Fig. 3C) were significantly different between groups according to the log-rank test ($\chi^2(3) = 17.9$, $p < 10^{-3}$).

Expression of immune and metabolic genes

Repeated exposure to *A. astaci* and different food availability significantly affected the expression of the metabolic genes CS ($H(3) = 15.34$, $p = 0.002$) and GAPDH ($H(3) = 9.72$, $p = 0.021$), and the immune gene C/EBP- β ($H(3) = 14.54$, $p = 0.002$), with no significant effects on ProPO expression (Fig. 4A; Suppl. material 1: Table S5.4A, C, E, G). The expression of metabolic genes was reduced in groups fed once a week, but the significantly lower mean rank was confirmed by post-hoc analysis only for the infected group (CS: $p = 0.01$; GAPDH: $p = 0.038$; Suppl. material 1: Table S5.4B, D). Conversely, the expression of the immune gene C/EBP- β was elevated in the groups fed once a week, but again, a significantly higher mean rank was confirmed by post-hoc analysis only for the infected group ($p = 0.007$; Suppl. material 1: Table S5.4F).

Relationships amongst gene expression variables were further analysed using the principal component analysis. Two principal components captured most of the variability in the dataset (87.6%, Fig. 4B), justifying the reduction of the complexity of the data analysis from four dimensions to two dimensions. The first principal component

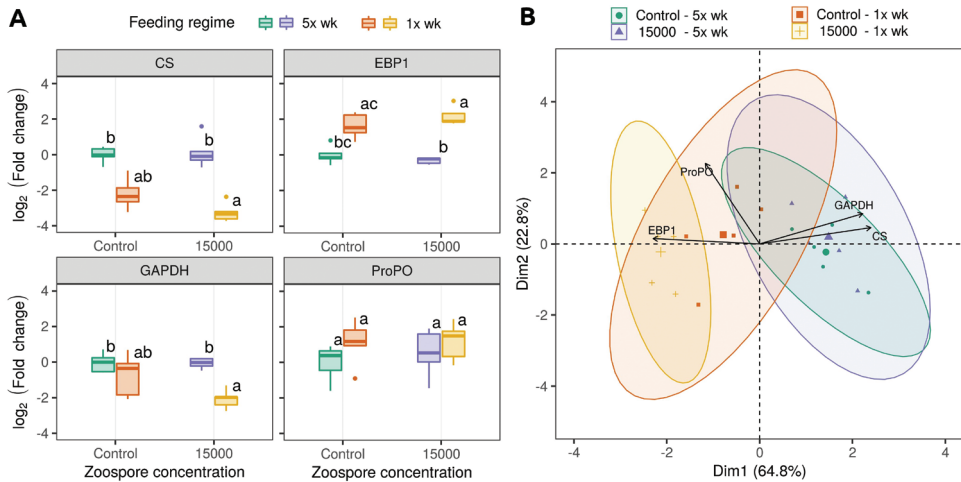


Figure 4. Differential expression of immune and metabolic genes in Experiment 2 analysed using **A** inferential comparison between groups and **B** principal component analysis (PCA). Different letters in panel A denote significant differences.

is strongly correlated with three of the gene expression variables CS, GAPDH and C/EBP- β , suggesting that these variables may vary together. PCA analysis also showed a high positive correlation between metabolic gene expressions, a positive correlation between immune gene expressions and negative correlations between metabolic and immune gene expressions. Consistent with the inferential analysis, experimental groups fed five times a week showed higher expression of GAPDH and CS genes.

Energy cost of infection

The food-restricted infected group of Experiment 2 had to activate and maintain immune response to the pathogen, inducing additional energy costs in contrast to the respective control (non-infected) group. Based on the average moisture content of the hepatopancreas, crayfish had 33.357 kJ/g in reserve. The comparison of total weight change between the two groups showed a total difference of 0.025 g, corresponding to 12.07 J/day of energy reserves used for immune response maintenance in infected individuals weighing on average 0.3 g.

Discussion

Chronic infection of invasive marbled crayfish juveniles with the *A. astaci* pathogen leads to trade-offs in energy use that reduces growth. Although this is the first such finding for crayfish, this is consistent with previous research on other invertebrates (i.e. arthropods: Rantala and Roff 2005; Bascuñán-García et al. 2010; Körner et al. 2017;

molluscs: O'Connell-Milne et al. 2016) and vertebrates (Lochmiller and Deerenberg 2000; Lee 2006; van Der Most et al. 2011; Bonneaud et al. 2012), which suggest that maintenance of an active immune system is energetically demanding, and trade-offs against growth, reproduction and development following infection are frequent. However, effects on growth observed here were achieved under repeated infections with high pathogen loads, while the observed sublethal effects (reduction of growth rates) translated into lethal effects (mortality) only under extreme food limitation. This shows that the marbled crayfish is (i) highly resistant to one of the most virulent haplogroups of *A. astaci* pathogen (haplogroup B) and can act as its carrier, and (ii) requires multiple stressors occurring simultaneously to suffer increased mortality.

Growth was reduced under both pathogen concentrations in Experiment 1 and under both feeding regimes in Experiment 2. Even though not statistically significant in some scenarios, the omnipresence of effects on growth suggest that exposure to pathogens increases allocation of energy to the immune system. Alternatively, the observed slower growth of infected groups could be the consequence of suppressed food intake suggested to occur during immune challenges (Lochmiller and Deerenberg 2000; Moberg 2000). We deem this unlikely because food was entirely consumed by crayfish in our experiments, suggesting that observed effects on growth are, indeed, related to the energy allocation to immune response.

A larger effect size of repeated infections on weight was observed in both experiments and became especially evident in Experiment 2. Here, the extreme food limitation (feeding once a week) almost completely ceased growth in both control (non-infected) and infected groups. The miniscule (if any) weight increase of the control food-restricted group suggests that those individuals were very near or at the point of starvation. Additional need to fuel the immune response of infected individuals further depleted energy reserves, thus increasing the severity of starvation resulting in increased mortality of the infected group fed once a week.

Starvation, as well as the difference in severity of starvation, was reflected in the down-regulation of genes involved in the central metabolic pathways of glycolysis and citrate cycle in both infected and non-infected group fed once a week. This is in line with previous studies on freshwater species which have shown that the expression of both CS and GAPDH decrease during starvation and fasting (Tripathi and Verma 2003; Salem et al. 2007). The most pronounced reduction in metabolic gene expression was observed in the food-restricted infected group, corroborating our assumptions that immune response to a pathogen resulted in additional depletion of energy reserves.

Food limitation and the resulting starvation induced up-regulation of the C/EBP- β transcription factor, belonging to the CCAAT/enhancer-binding protein (C/EBP) family, which are involved in the regulation of the metabolism, cell growth, differentiation, death, immune and inflammatory processes (Ramji and Foka 2002; Wang et al. 2019). C/EBP- β up-regulation is crucial in the acute phase of the *A. astaci* infection in the native noble crayfish (Boštjančić et al. 2021). In our study, C/EBP- β was up-regulated in both control and infected food-restricted groups in comparison to groups fed five times a week, with the highest change in C/EBP- β expression observed in the food-restricted

infected group. This indicates that stressful conditions of both starvation and pathogen infection increase the activity of C/EBP- β gene, signifying its role in regulation of both metabolism and immune response in crayfish. The expression of the ProPO gene, which belongs to the core immune response mechanism in crustaceans engaged in the encapsulation of the pathogen (ProPO pathway; Cerenius et al. 2003), did not differ significantly between infected and non-infected individuals in any of the groups in Experiment 2. This is consistent with previous reports from infection trials that applied a single infection using lower pathogen concentration of the same *A. astaci* haplogroup (Boštjančić et al. 2021), suggesting that there was no acute immune response and that energy was not invested into synthesis of immune effectors in hepatocytes. Rather, it probably went into the reparation of the damaged epicuticle, as the first line in the defence against the pathogen (Cerenius et al. 2003), which is energetically costly.

Lower mortality in the group fed five times a week indicates that these individuals were more able to cover the additional energy costs of fighting the disease, including the potential reparation of damage, as opposed to the group fed once a week. The scale of the cost is well represented by the differences in weight gain between the infected and non-infected food-restricted groups: the 'missing' weight can be solely attributed to energy costs of fighting the infection (immune response maintenance costs) and related consequences.

Chronic exposure to the *A. astaci* pathogen is unlikely to have a long-term effect on marbled crayfish populations, except under extreme limitation of food availability, which was used in this study as a proxy for density-dependent effects. In all experimental groups, infected individuals grew more slowly than non-infected. In nature, the slower growth could translate into slower maturation rates and/or lower fecundity of infected individuals due to size-dependence of these traits (Hossain et al. 2019) and reduction in energy available for reproduction. However, we do not expect that such growth reduction would have high implications for the invasion success of this species due to its parthenogenetic mode of reproduction and high fecundity in comparison to other native and invasive crayfish (Hossain et al. 2018).

Furthermore, a lower proportion (40 - 55%) of individuals tested positive for *A. astaci* in Experiment 1 (analyses performed six weeks after last infection) compared to Experiment 2 (analyses performed two weeks after the last infection), where almost all individuals (87 - 100%) were *A. astaci* positive. This either means that: i) infections were less successful in Experiment 1 or that ii) some individuals from Experiment 1 were able to contain the infection during the six weeks since the last *A. astaci* infection, unlike in Experiment 2 where *A. astaci* detection was performed two weeks after the last infection. We consider the former unlikely, as the procedures were the same in both experimental trials and number of infections was higher in Experiment 1 (6 repeated infections in Experiment 1 vs. 5 in Experiment 2). If the latter is true, this suggests that at least some proportion of marbled crayfish that survived repeated infections could potentially efficiently contain the pathogen and minimise further trade-offs with growth.

Our results add to the growing knowledge regarding the high tolerance of marbled crayfish to multiple single and combined stressors (Kaldre et al. 2015; Guo et al.

2019; Hossain et al. 2021; Stara et al. 2021). Such tolerance, in combination with fast growth, high fecundity and parthenogenetic mode of reproduction, prime it for becoming one of the most successful crayfish invaders.

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Supplementary material I

Protocols, primers, statistical support data, images

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Explanation note: The file contains additional detailed descriptions of used protocols and applied statistical analyses, additional statistical support data and images.

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Jack, master or both? The invasive ladybird *Harmonia axyridis* performs better than a native coccinellid despite divergent trait plasticity

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Abstract

The plasticity of performance traits can promote the success of biological invasions and therefore, precisely estimating trait reaction norms can help to predict the establishment and persistence of introduced species in novel habitats. Most studies focus only on a reduced set of traits and rarely include trait variability that may be vital to predicting establishment success. Here, using a split-brood full-sib design, we acclimated the globally invasive ladybird *Harmonia axyridis* and a native co-occurring and competing species *Cheilomenes lunata* to cold, medium and warm temperature regimes, and measured critical thermal limits, life-history traits, and starvation resistance. We used the conceptual framework of “Jack, Master or both” to test predictions regarding performance differences of these two species. The native *C. lunata* had a higher thermal plasticity of starvation resistance and a higher upper thermal tolerance than *H. axyridis*. By contrast, *H. axyridis* had a higher performance than *C. lunata* for preoviposition period, fecundity and adult emergence from pupae. We combined trait responses, transport duration and propagule pressure to predict the size of the populations established in a novel site following cold, medium and warm scenarios. Although *C. lunata* initially had a higher performance than the invasive species during transport, more individuals of *H. axyridis* survived in all simulated environments due to the combined life-history responses, and in particular, higher fecundity. Despite an increased starvation mortality in the warm scenario, given a sufficient propagule size, *H. axyridis* successfully established. This study underscores how the combination and plasticity of multiple performance traits can strongly influence establishment potential of species introduced into novel environments.

Keywords

Acclimation, biological invasions, climate change, Coccinellidae, population growth, temperature tolerance

Introduction

The establishment and spread of invasive species in novel habitats have been attributed to factors pertaining to human dimensions such as propagule pressure and how and where these propagules have been introduced or moved, and to natural drivers, including the invasibility of the habitat and the specific traits of the invasive species (Hayes and Barry 2008; van Kleunen et al. 2010a; Blackburn et al. 2018; Enders et al. 2020). In particular, phenotypic plasticity, defined as the ability of a genotype to produce different phenotypes when exposed to varying conditions within an organism's lifetime, is often cited as enabling alien species to rapidly respond to novel or varying conditions, increasing their invasion potential (Baker 1965; Richards et al. 2006; Colautti et al. 2017; Torchyk and Jeschke 2018). Three patterns of response have been proposed to describe the reaction norms or performance curves that could benefit an invasive species compared to a native counterpart in the introduced range (Richards et al. 2006). First, the invasive species maintains fitness more consistently than the native species across environmental conditions, including stressful ones (also known as a “General-purpose” or “Jack-of-all-trades” phenotype). Second, the invasive species has a higher fitness in the most favourable conditions compared to the native one (“Master-of-some” phenotype). Finally, the invasive species has both a broader and a higher peak performance than the non-invasive species across varied environmental conditions (“Jack-and-master”).

In plants, the extent of phenotypic plasticity can be greater in invasive alien species than native species, especially for key performance traits, but plasticity does not always translate into increased fitness (for a review see Davidson et al. 2011; Gallagher et al. 2015; Huang et al. 2015). Other studies, including meta-analyses, have found no support for increased plasticity in invasive species (Godoy et al. 2011; Palacio-López and Gianoli 2011). Although the study of plasticity in invasive insects is more limited than in plants, data suggest that life-history traits and temperature tolerance of invasive insects and terrestrial arthropods are generally more plastic than their native counterparts (e.g. Slabber et al. 2007; Janion et al. 2010; Nyamukondiwa et al. 2010; Weldon et al. 2011; Coccia et al. 2013; Mutamiswa et al. 2018). However, as for plants, a lack of support is also found in insects and springtails (Gibert et al. 2016; Janion-Scheepers et al. 2018; Phillips et al. 2020; Da Silva et al. 2021). The absence of a general pattern of increased plasticity in invasive species may stem from several factors: plasticity differences between native and alien insects can depend on the trait examined, the invasive and native species compared, the timing of the comparison during the invasion process, the experimental conditions and methodology used, as well as conditions and characteristics of the environment (Slabber et al. 2007; Terblanche et al. 2010; Nyamukondiwa et al. 2018; Weldon et al. 2018). Different combinations of plastic and non-plastic responses of traits, both morphological and physiological, can compound into flat or advantageous fitness reaction norms. Therefore, the extent of trait- and context-specific differences in reaction norms calls for examining multiple, rather than single, traits between closely-related or ecologically-equivalent species.

Furthermore, given the dependence of insects to surrounding conditions to maintain body temperature and the continuing change in temperature means and extremes associated with climate change (Arias et al. 2021), a focus on trait responses to different temperature exposures is crucial. This approach can highlight differences in the thermal plasticity of traits that underlie individual fitness and improve predictions of population persistence in new and changing conditions (Chun et al. 2007; Morris et al. 2011; Tomlinson et al. 2015; Da Silva et al. 2021).

Several abiotic and biotic filters are encountered during and following colonization of species into a new environment (Catford et al. 2009). Typically, the invasion process is divided into several stages (transport, introduction, establishment and spread) with barriers that species need to overcome to move across stages (Blackburn et al. 2011). Some species possess characteristics that can enhance the successful transition across stages (Hayes and Barry 2008; Blackburn et al. 2011). For insects, most species are introduced as contaminants of imported products, or stowaways on airplanes and ships, as opposed to deliberate introductions, except for biocontrol agents (Kiritani and Yamamura 2003; Work et al. 2005; Hulme et al. 2008; Liebhold et al. 2006, 2012; Faulkner et al. 2016). Survival at the transport stage can rely heavily on the ability to tolerate conditions in the transport vessel, while the ability to cope with food shortages through starvation resistance will greatly increase the chances of survival (Renault et al. 2018). Once introduced, life-history traits such as developmental time and fecundity, and other performance-related traits including resource acquisition, competition ability and stress tolerance, can also assist species to establish and produce viable populations. Dispersal ability, and a wide tolerance and diet breadth can then promote spread into new environments (Hayes and Barry 2008; Blackburn et al. 2011). Thus, a combination of traits involved within and across the multi-stage invasion process is important for successful invasion, yet studies rarely consider how the combination of stress resistance, life-history and dispersal traits may impact species' overall survival and persistence in new environments (Renault et al. 2018).

Studies that model the dynamics of insect invasions often focus on the establishment and spread stages. Typically, these studies integrate population growth, population size, dispersal rate (e.g. Miller and Tenhumberg 2010; Hui and Richardson 2017; Lux 2018) and/or functional traits (such as thermal tolerance; e.g. Kearney et al. 2009; Hartley et al. 2010) to determine species' persistence and distribution. However, small numbers of starting propagules and trait responses to conditions along the pathway can result in failed invasions (Zenni and Nuñez 2013). Therefore, considering the sequence of events during an insect invasion rather than focusing on a single stage, and modelling the consequences of multiple trait's variation and plasticity on species survival or performance across invasion stages, can portray a more informative picture of a species' potential to establish.

The harlequin ladybird beetle, *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae), is a notorious invasive species whose establishment and spread has been associated with the rapid decline of native species in multiple countries, likely as a result of intraguild predation and competition for resources (Roy et al. 2016). Life-history traits

such as a large body mass, multiple generations per year, high fecundity and longevity, fast developmental rate, and an altered sex ratio in favour of females have been proposed to promote its invasiveness (Bazzocchi et al. 2004; Labrie et al. 2006; Hemptinne et al. 2011; Roy et al. 2016). A broad thermal tolerance range, mostly stemming from the beetle's high cold hardiness, is likely to favour its persistence in novel conditions (Berkvens et al. 2010; Barahona-Segovia et al. 2016). Moreover, thermal acclimation effects of multiple life-history traits have been reported in *H. axyridis*, such as development (Grill et al. 1997; Berkvens et al. 2008; Knapp 2014), fecundity (Castro et al. 2011; Zhang et al. 2014), body size (Grill et al. 1997; Knapp and Nedved 2013), quiescence or diapause (Sakurai et al. 1992; Lombaert et al. 2008), melanism (Michie et al. 2011; Knapp and Nedved 2013), foraging behaviour (Mondor and Warren 2000) and upper thermal tolerance (but see Boher et al. 2018; Shinner et al. 2020). Establishing whether this plasticity benefits the invasive species to a greater extent than a native or non-invasive ladybird beetle in the introduced range can be influenced by several factors, such as the site of origin of the species, period in the invasive range since introduction, and the genetic history and diversity of the invasive and native populations (Hulme 2008, van Kleunen et al. 2010b). However, useful native and invasive insect comparisons stem from assessing co-occurring species from similar taxonomic families or ecological niches (e.g. Tomlinson et al. 2015; Da Silva et al. 2021). Basal traits and their thermal plasticity can be used to simulate scenarios of establishment and spread in novel environments for both species, successful alien and native species, and predict their population dynamics and survival in different thermal conditions (van Kleunen et al. 2011; and see Brass et al. 2020 for complementary of stage-structured models to plasticity data).

In this study, we first examine trait responses of *H. axyridis* and a sympatric and often syntopic native species, *Cheilomenes lunata* (Fabricius, 1775) (Coleoptera: Coccinellidae), to three ecologically relevant temperature regimes (cold, medium and warm). We test if the patterns of phenotypic response to temperature in these species are in line with the “Jack-of-all-trades”, “Master-of-some” or “Jack-and-Master” models as depicted in Fig. 1. While comparing two species has been highlighted as limiting the ability to make adaptive inferences of phenotypic variation (e.g. Felsenstein 1985; Garland and Adolph 1994), here, we do not aim to infer adaptation nor to describe the specific drivers of these differences. Instead, we test specific model predictions using an invasive and a co-occurring native coccinellid and assess the magnitude and direction of responses to temperature for multiple traits in these species (Kelley et al. 2014). We targeted traits likely to be important in determining successful transport, establishment and spread, including starvation resistance, thermal tolerance limits and life-history traits as fitness proxies. We then use a computer simulation to combine propagule size and trait responses measured in the study to determine the number of beetles per species that would establish after crossing multiple stages of invasion at three scenarios of temperature conditions and assess if these species' specific responses would facilitate their survival in these environments. Given the focus on temperature effects, our goals should also provide insights into potential responses of these species to contemporary climate warming, a facet that has been neglected for native coccinellid species (Sloggett 2021).

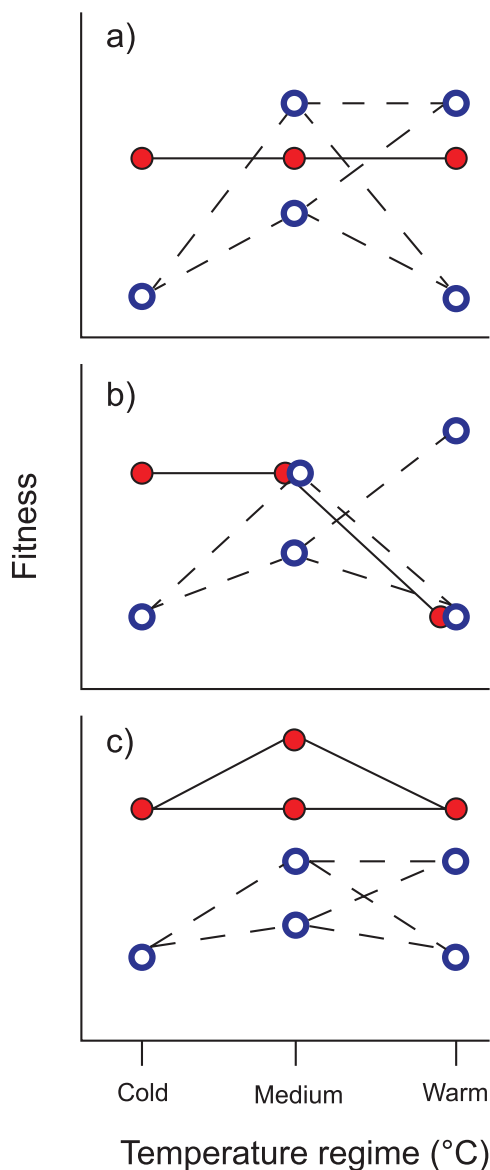


Figure 1. Theoretical expectations for the fitness responses of the study species at three temperatures. The invasive species *H. axyridis* (solid circles) follows one of three patterns **a** Jack-of-all-trades: *H. axyridis* is able to maintain fitness in all environmental conditions, including sub-optimal or stressful ones (cold and warm). The native *C. lunata* (empty circles) may outperform the invasive in some conditions **b** Master-of-some: *H. axyridis* is better able to increase fitness in favourable conditions and, typically, more so than the native **c** Jack-and-master: *H. axyridis* is able to maintain fitness and perform better than its native counterpart. The medium regime is assumed to be the most favourable environment for both species but given the lack of literature for the native species, optimal temperatures for fitness could correspond to the medium or the warm regime since information about its thermal optimum is limited (see text). Diverse lines of reaction norms within species means that multiple trajectories are possible.

Methods

Study species and field collections

Harmonia axyridis was first recorded in the south-western region of the Western Cape Province, South Africa, in 2001 (Stals 2010; Roy et al. 2016). It rapidly spread, occupying six of the seven biomes in the country, and extending from sea-level to > 1800m in elevation (Stals and Prinsloo 2007; Stals 2010). Although native to Asia, molecular data suggest that *H. axyridis* was introduced to South Africa from an invasive source population in eastern North America (Lombaert et al. 2010; Collop et al. in prep) and is now found in most urban and semi-urban habitats in the country. The native species, *Cheilomenes lunata*, is smaller than *H. axyridis* (from this study, mass (mg) = 16.3 ± 4.5 (n = 133) and 20.4 ± 4.7 (n = 112), for *C. lunata* and *H. axyridis*, respectively) and is ubiquitous in Southern Africa and some parts of Eastern and Central Africa (Picker et al. 2004; iNaturalist.org). There is currently limited information on its physiology and life-history. Both species feed primarily on aphids and are frequently found on the same plants. In South Africa, *H. axyridis* has a critical thermal maximum of -44°C (Shinner et al. 2020), and from data gathered in other regions, its development, fecundity and survival typically decline at temperatures above 30°C (de Oliveira Ramos et al. 2014; Zhang et al. 2014; Barahona-Segovia et al. 2016; Roy et al. 2016). Most of this species' life-history traits have optimal temperatures between 20 and 25°C (Suppl. material 1: table S1).

Individuals of *H. axyridis* and *C. lunata* were collected from 10 different locations around Stellenbosch, South Africa, between February and May in 2014 and 2015 (Suppl. material 1: table S2). Similar sample sizes were collected across sites to enhance genetic diversity and avoid inbreeding effects. Beetles were collected from rose plants in urban gardens where both species co-occur and feed mostly on rose aphids, *Macrosiphon rosae* (Linnaeus, 1758).

Rearing and temperature regimes

For each species, stock populations (n = 200) were maintained in temperature-controlled chambers (SANYO MIR-254, SANYO Electric Co., Ltd; Osaka, Japan) with a summer photoperiod of 14L:10D, and a temperature cycle of $25.5 \pm 0.1^\circ\text{C}$ for 18 h (day), $18.5 \pm 0.1^\circ\text{C}$ for 6 h (night) (mean temperature of $23.8 \pm 3.0^\circ\text{C}$). This regime resembles microsite temperature profiles experienced in Stellenbosch during peak ladybird beetle abundance (i.e. March to June; Chidawanyika and Terblanche 2011 and field data), and is within the range of optimal temperatures reported for different traits of *H. axyridis* (Suppl. material 1: table S1).

Stock populations (F0) of each species were kept in 2-L plastic containers covered with mesh for ventilation (< 50 individuals per box to avoid crowding). Beetles were given live aphids *ad libitum* (~30 individuals/beetle from rose aphids *M. rosae*, oak aphids *Tuberculatus annulatus* (Hartig, 1841) and Russian wheat aphids *Diuraphis noxia* (Kurdjumov, 1913)) every three days and 1:10 honey:distilled water solution

ad libitum, and left to reproduce for one generation. F1 beetles were then reared in separate containers from field populations. Once mature, F1 females and males were chosen at random to form families ($n = 50\text{--}60$ unique pairs per species). Twenty-five to 30 F1 mating pairs (or families) were placed in individual 9-cm Petri dishes and fed 20 live aphids daily (as described above) with honey solution *ad lib*. When an egg clutch was laid, parents were moved to a different Petri dish to avoid cannibalism. At hatching (~ 3 days), F2 larvae were divided as < 10 individuals per Petri dish. Larvae were given frozen rose aphids and honey solution daily. Rose aphids were collected in the field but frozen at -80°C prior to experiments to have high quantities of consistent food during development. Pilot trials showed that larvae from both species readily consume frozen aphids. Larvae were moved to individual Petri dishes when pre-pupae first appeared to prevent cannibalism. At emergence from pupae (~ 5 days), adults were placed in individual dishes and given honey solution *ad lib*. Adults were weighed 24 h after emergence and then fed 10 frozen rose aphids (see experimental design in Suppl. material 1: fig. S1).

F2 adults from each family (full-sibs) were then equally spread across three temperature treatments: cold ($20.5 \pm 0.1^\circ\text{C}$ for 18 h and $13.3 \pm 0.1^\circ\text{C}$ for 6 h; mean temp = $18.7 \pm 3.1^\circ\text{C}$), medium ($25.5 \pm 0.2^\circ\text{C}$ for 18 h and $18.5 \pm 0.1^\circ\text{C}$ for 6 h; mean temp = $23.9 \pm 3.0^\circ\text{C}$) and warm ($30.5 \pm 0.1^\circ\text{C}$ for 18 h and $23.3 \pm 0.1^\circ\text{C}$ for 6 h; mean temp = $28.8 \pm 3.1^\circ\text{C}$) two days after pupal emergence, which is when beetles are capable of flight (Hodek et al. 2012). These temperature regimes were selected given field microsite data and the optimal temperatures of multiple traits found in other studies for *H. axyridis* (Suppl. material 1: table S1). Optimal performance temperatures are not available for the native species, but studies on the congener *C. sexmaculata* (Fabricius, 1781) have shown that fecundity, egg hatching success, successful pupal emergence and adult lifespan is highest between 30 and 35°C (Wang et al. 2013). By contrast, Hodek et al. (2012) found that the net reproduction rate and lifetime egg production of 12 species of ladybird beetles peaked at 25°C . We therefore chose optimal and warm temperature regimes within this range ($20\text{--}30^\circ\text{C}$). The photoperiod was kept as in the maintenance section, and 20 frozen aphids and honey solution were provided daily for each treatment.

Sex was determined for *H. axyridis* following McCornack et al. (2007), and using abdominal morphology and colouration for *C. lunata* (concave last posterior segment in males and convex in females, darker labrum pigmentation in females; pers. comm. Riaan Stals).

Starvation resistance

Two days post-emergence, distilled water was provided, all food removed and beetles assigned to temperature treatments by splitting siblings equally across treatments (*H. axyridis*: $n = 36$ (cold), $n = 41$ (medium), $n = 35$ (warm); *C. lunata*: $n = 43$ (cold), $n = 37$ (medium), $n = 53$ (warm)). Beetles remained in treatments until they succumbed to starvation. Mortalities were checked twice daily (09:00, 18:00) and when

found, body mass was measured within 30 min, and sex determined. Starvation resistance was measured as the number of days in the treatment until death, and the percentage of mass loss between the start and end of starvation trials recorded.

Critical temperature limits (CTLs)

Beetles were kept in temperature treatments for 7–9 days prior to thermal tolerance trials. This acclimation period was chosen as it induces plastic effects in most insects (Weldon et al. 2011). Food was removed and distilled water provided a day prior to trials. Individuals were first weighed (Avery Berkel, UK, ± 0.0001 mg) and then placed inside a double-jacketed chamber and left to equilibrate for 15 min to a set temperature of 25 ± 0.2 °C controlled by a programmable water bath (Grant GP200-R4; Grant Instruments, England). The experimental temperature was monitored inside an empty central chamber using a thermocouple connected to a logger (TC-08, Pico Technology) and ramped up (critical thermal maximum; CT_{max}) or down (critical thermal minimum; CT_{min}) at a rate of 0.1 °C·min⁻¹. This rate was chosen to avoid hardening effects induced by rates that are too slow and to avoid missing the end-point if rates are too fast. Beetles were checked every 30 min for the first 2 h, then every 10 min until vigorous movement (CT_{max}) or loss of righting response (CT_{min}), followed by 2–3 min checks until set thresholds. CT_{min} was reached when there was no visible movement of the head, antennae or legs after prodding (*H. axyridis*: $n = 51$ (cold), $n = 44$ (medium), $n = 48$ (warm); *C. lunata*: $n = 40$ (cold), $n = 37$ (medium), $n = 37$ (warm)). CT_{max} was reached when beetles no longer grabbed the end of a thin rod after gentle prodding, indicating a loss of muscle control (*H. axyridis*: $n = 51$ (cold), $n = 51$ (medium), $n = 54$ (warm); *C. lunata*: $n = 38$ (cold), $n = 45$ (medium), $n = 45$ (warm)). Beetles were always checked in the same order. Since some ladybird beetles are known to feign death and inactivity, individuals had to display physiological endpoints three times in a row with the first reading taken as the endpoint. At the end of the trials, beetles were weighed, placed in a -20 °C freezer and stored in 99% ethanol.

Life-history traits

After 7 days in respective temperature treatments, F2 females (*H. axyridis*: $n = 21$ (cold), $n = 19$ (medium), $n = 24$ (warm); *C. lunata*: $n = 17$ (cold), $n = 19$ (medium), $n = 21$ (warm)) were paired up with F2 males (that originated from different families but that had been exposed to the same temperature regime) to create mating pairs. The pre-oviposition period (i.e. number of days from pairing to first egg clutch) and number of eggs laid were recorded for 2 weeks.

Three randomly-chosen clutches per mating pair, maintained at the same temperature regimes as their respective parental F2 pairs, were checked twice daily for hatched larvae (fully emerged from egg casings) until a day after eggs started hatching. In addition, the number of yellow and black eggs were recorded. Yellow eggs are assumed to be trophic (unfertilised) eggs while black eggs are typically fertilised eggs that did not

hatch (Perry and Roitberg 2005). Hatching success was calculated as the percentage of total number of hatched larvae from the total number of viable eggs (which included hatched larvae and fertilised eggs).

Larvae (F3) were placed in individual Petri dishes and monitored until emergence from pupae with developmental time set as the number of days from egg (Day 0) to successful emergence from pupae. Mating pairs had to at least produce three live larvae to be included in analyses which resulted in the exclusion of a single pair. Beetles (F3) were given honey solution, weighed 24–36 h after eclosion, and sex determined five days after eclosion to allow for hardened elytra. The percentage of adult beetles that successfully emerged from pupae was calculated per mating pair as the total number of successfully emerged adults over the total number of pupae monitored.

Larvae that died within a day of being transferred to Petri dishes were assumed to have died from the transfer process and were discounted from calculations (<3%). Mating pairs and larvae were fed 20 live aphids daily (rose, oak or Russian wheat aphids) and provided with honey solution. To ensure that mating adults had not lost body condition, body mass was recorded before and after mating periods.

Intrinsic rate of population increase (r) was determined as $r = (\ln R_0) / T_g$, where the net reproductive rate (R_0) (Birch 1948) was defined as

$$R_0 = \sum l_x m_x$$

where x is the age of the female in days from the day of emergence from pupa until the end of data collection at $x = 23$ days, l_x is the probability (0 to 1) of being alive at age x , and m_x the number of females produced by each female at age x . Since only those females that survived the trial were used in the analysis, l_x was set to 1 for all values of x (Dillon et al. 2007). Therefore, the net reproductive rate was the expected number of F3 females produced by an F2 female and reflects the maximum reproductive rate (since mortality was not included in the calculation). We used the mean development time of F3 larvae as a proxy for the generation time T_g of F2 females.

Statistical analysis

For the analysis of starvation resistance, we first drew Kaplan-Meier survival curves to illustrate survival probability over time (in days) (survival package; Therneau and Lumley 2018) using R Statistical software v. 3.6.3 (R Core Team 2017). We then used a Cox-proportional hazards model to test for the effects of percentage of mass loss, sex, species and temperature treatment on survival (coxme package; Therneau 2018). Family ID of individuals used in trials was included as a random effect.

For all other traits (CT_{min} , CT_{max} , preoviposition period, total eggs produced, hatching success, developmental time, pupal emergence success, and intrinsic rate of increase), we constructed full general or generalized linear mixed effects models comprising mass, sex, species, treatment and their interactions as predictors, and a random effect of family

ID. We compared each full model with a model without the random effect using the nlme package (Pinheiro et al. 2016) following Zuur et al. (2009). As body mass and sex can predict life-history, starvation resistance and tolerance traits of beetles and other insects (e.g. Weldon et al. 2011; Lancaster et al. 2015; Barahona-Segovia et al. 2016; Raak-van den Berg et al. 2017; Nyamukondiwa et al. 2018), we included these variables in our statistical models. An appropriate variance structure was used for critical thermal limits to improve model fit (Zuur et al. 2009). For preoviposition period, total eggs laid and developmental time, we used a Poisson error distribution while for egg hatching success and pupal emergence success, we fitted the model using a binomial error distribution. The mass of the female was included in models of preoviposition, total eggs, and intrinsic rate of increase, while the mass prior to the trial was included as predictor for critical limits and adult mass was included in models predicting developmental time. Model outputs were also compared to those of mixed-effects models constructed using the lme4 package (Bates et al. 2015). Backward selection of non-significant terms was used until a minimum adequate model was obtained using maximum likelihood. Interpretations were based on the parameter estimates of the most parsimonious model. Plots of fitted vs standardised residuals and histograms of standardised residuals were checked for violations of model assumptions. Full model structure and best model outputs for all traits are provided in Suppl. material 1: table S4. Interpretations of significant interactions were made by plotting model estimates (effects package; Fox 2003). Pairwise differences of trait means between species and/or sexes within treatments were assessed using least squares means (lsmeans package; Lenth 2016). Trait original means \pm SD and sample sizes are provided in Suppl. material 1: table S5.

In addition, for each trait, candidate models that had a Δ AICc value of < 2 were used in model averaging (Burnham and Anderson 2002; Grueber et al. 2011). We used the full-average method to determine parameter coefficients of the averaged model (Burnham and Anderson 2002), and the 'model.avg' function in the MuMIn package (Barton 2018). Model averaging was performed for CT_{min} , CT_{max} , developmental time and egg hatching success. Averaged model outputs did not change results and are therefore only provided in supplementary materials (Suppl. material 1: table S6).

Modelling population size across invasion stages

Using R, we simulated the effect of trait combination on population size for each species using an invasion framework that includes the transport and establishment stages. We assumed that starvation resistance, life-history traits and upper thermal tolerance shaped individual persistence through transport and establishment. The model simulated the case where a number of individuals were transported to a new habitat with starvation resistance shaping the number of survivors through time. These survivors could establish in the new habitat and produce a new generation. Temperature scenarios (cold, medium or warm) were assumed to be fixed across transport and establishment stages.

We used experimental data to model survival numbers at each stage of the journey. We calculated the proportion of individuals that survived transportation based on the

starvation resistance data. Specifically, we counted the number of individuals that died each day from 1 to 20 days at 0.5-day increments (the resolution of the starvation resistance data), with all individuals surviving on day 1 and all succumbing to starvation in 20 days. We fitted a Gompertz sigmoid growth function (Gompertz 1825; Tjørve and Tjørve 2017) to cumulative mortality count data for each species within each treatment: Mortality count = $a \cdot \text{Exp}(-\text{Exp}(-c(t-b/c)))$, where a represents the total number of beetles measured in each treatment per species, t time (in 0.5-day increments), c is the mortality change rate, and b/c is the time when mortality peaks. The simulation started with a randomly-chosen propagule size (between 1 and 100 adult beetles) transported to a new area with no access to food, and a randomly-selected period of travelling time between 1 and 20 days (at 0.5-day increments). Propagule size was multiplied by the survival proportion to determine the total number of beetles that survived transportation. Upon arrival in the novel environment, we assumed a 50% sex ratio of surviving and reproducing beetles (the sex ratio of both species in our experiments varied from 45.1 to 53.6% across treatments, with an average of 51.5%). To determine the number of beetles that successfully established in the new habitat, we calculated the product of the number of surviving females \times (i) the number of eggs laid \times (ii) the probability of viable eggs \times (iii) the probability of a viable egg that successfully hatched \times (iv) the probability of a larva that survived to adulthood. Parameters (i-iv) were randomly sampled from the corresponding probability density distributions based on the experimental data for each temperature treatment. The model ran for 1000 iterations for each species and each temperature scenario.

Data resources

Supplementary material is available at <https://doi.org/10.3897/neobiota.@@.91402.suppl1> and original data can be requested from the corresponding author.

Results

Parental family affiliation was important for traits of temperature tolerance, starvation resistance and life history ($\Delta\text{AICc} > 2$ for models with versus without Family ID as a random effect) except for hatching success, pupal emergence success and intrinsic rate of increase.

Starvation resistance

Starvation resistance of both species increased as the temperature of treatments decreased ($p < 0.0001$; Fig. 2). However, starvation resistance of the native *C. lunata* increased more between the warm and cold treatments than for the alien *H. axyridis* (from 4 days to 11 days and from 4.5 days to 9 days, respectively; species \times treatment interaction, Suppl. material 1: table S3). Overall, the relationship between the percentage mass loss during the starvation trial differed between sexes and across treatments

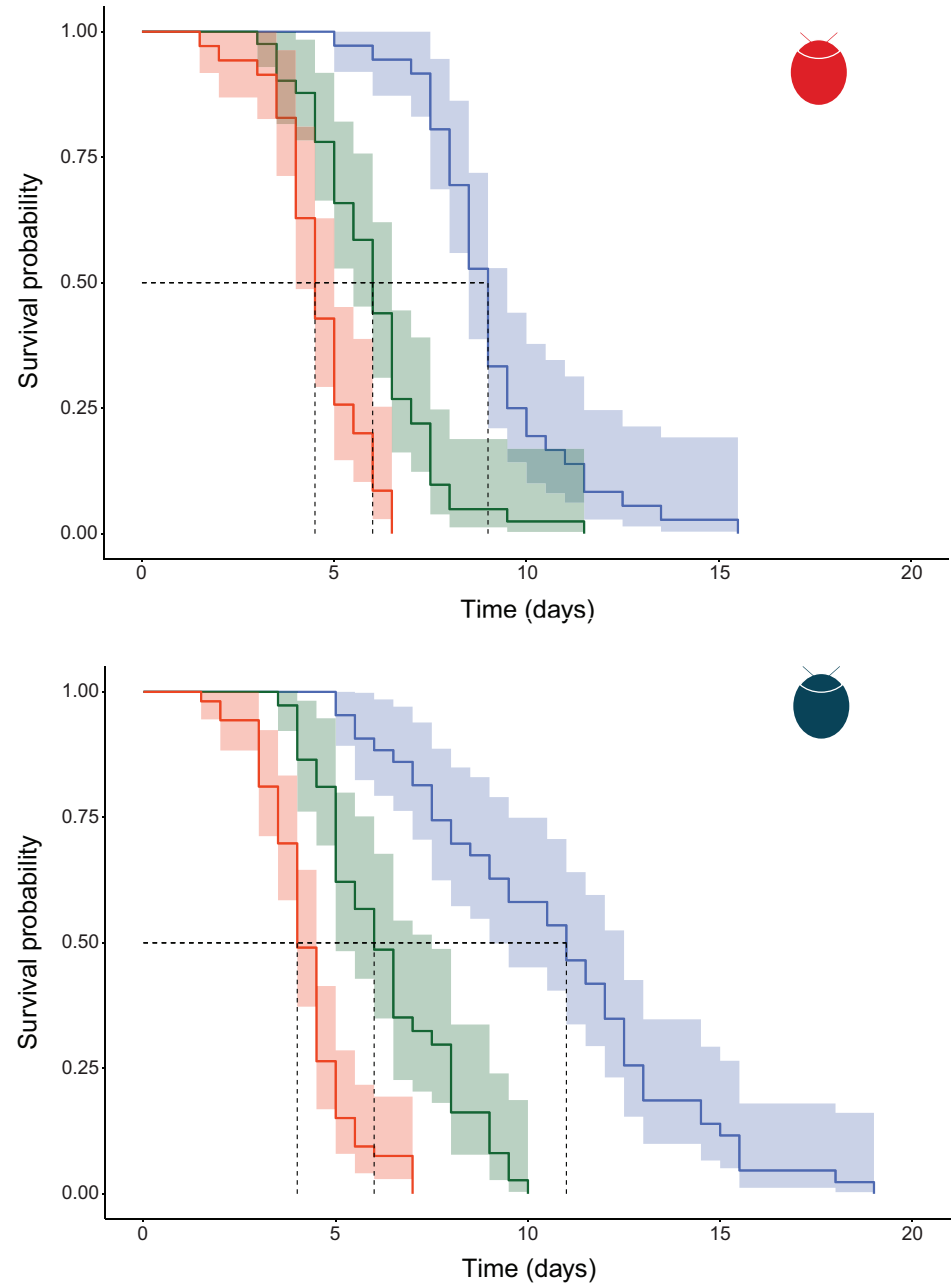


Figure 2. Starvation resistance of the invasive *Harmonia axyridis* and native *Cheilomenes lunata* kept at three temperatures. Kaplan-Meier survival curves were used to plot survival times of *H. axyridis* (top) and *C. lunata* (bottom) maintained at the cold (right - blue), medium (middle - green) and warm (left - orange) temperature treatments. Dotted lines represent 50% survival probability per treatment and corresponding numbers of days.

(significant three-way interaction among % mass loss \times sex \times treatment; Suppl. material 1: table S3, fig. S2), irrespective of species. In beetles that lost less body mass, females survived longer than males in the cold (10.5 days and 8.5 days, respectively) and medium treatments (6.5 days and 6 days, respectively), although both sexes survived for the same amount of time in the warm treatment (i.e. 4 days). For beetles that lost more body mass, females survived longer than males in the cold (11 days and 8.5 days, respectively) and medium treatments (7 days and 5.5 days, respectively), but for a shorter period in the warm treatment (females: 4 days and males: 5 days) (Suppl. material 1: fig. S2).

Thermal tolerance

The critical thermal minimum, CT_{min} , did not differ between species. Overall, CT_{min} was higher in beetles maintained in the warm ($1.0 \pm 1.4^\circ\text{C}$, $n = 85$) than cold treatment ($0.3 \pm 1.6^\circ\text{C}$, $n = 91$) ($p < 0.001$), and larger beetles had a lower CT_{min} ($p = 0.004$).

For CT_{max} , there was a significant interaction between mass, sex, and species. In *H. axyridis*, the CT_{max} of males and females increased with body mass, whereas, for *C. lunata*, CT_{max} of females did not vary with mass, but larger males had a higher CT_{max} than smaller males (mass \times sex \times species interaction, Fig. 3a). CT_{max} of females across treatments varied more than CT_{max} of males (sex \times treatment interaction; Suppl. material 1: fig. S3); for females, CT_{max} increased across all temperature treatments ($p < 0.01$ for all treatment comparisons), whereas CT_{max} of males only increased between cold and warm ($p < 0.001$), and between medium and warm treatments ($p = 0.003$). Overall, *C. lunata* had a higher CT_{max} than *H. axyridis* ($45.4 \pm 0.8^\circ\text{C}$ ($n = 128$) and $44.8 \pm 0.5^\circ\text{C}$ ($n = 156$), respectively; $p = 0.02$), and males had a higher CT_{max} than females in both species ($p < 0.01$). In both species, CT_{max} increased with temperature ($p < 0.01$).

Life-history traits

For both species, the preoviposition period decreased with temperature treatment ($p < 0.05$) but *H. axyridis* (3.9 ± 2.0 days, $n = 64$) had a shorter preoviposition period than *C. lunata* (5.5 ± 4.7 days, $n = 57$) ($p < 0.0001$; Suppl. material 1: table S3).

The relationship between total number of eggs and the mass of females differed across treatments and between species, which explains some of the variation in the total number of eggs produced (species \times treatment \times female mass interaction; Suppl. material 1: fig. S4). *Harmonia axyridis* laid consistently more eggs as temperature increased ($p < 0.0001$ for all comparisons), whereas *C. lunata* only had a lower number of eggs in the cold treatment ($p < 0.0001$; species \times temperature interaction; Fig. 3b). *Harmonia axyridis* laid more eggs than *C. lunata* in all treatments ($p < 0.0001$ for all comparisons).

Hatching success did not differ between species or treatments. The developmental time (from egg to pupal emergence) decreased with temperature ($p < 0.0001$ for all comparisons), but *H. axyridis* had a steeper change in developmental time

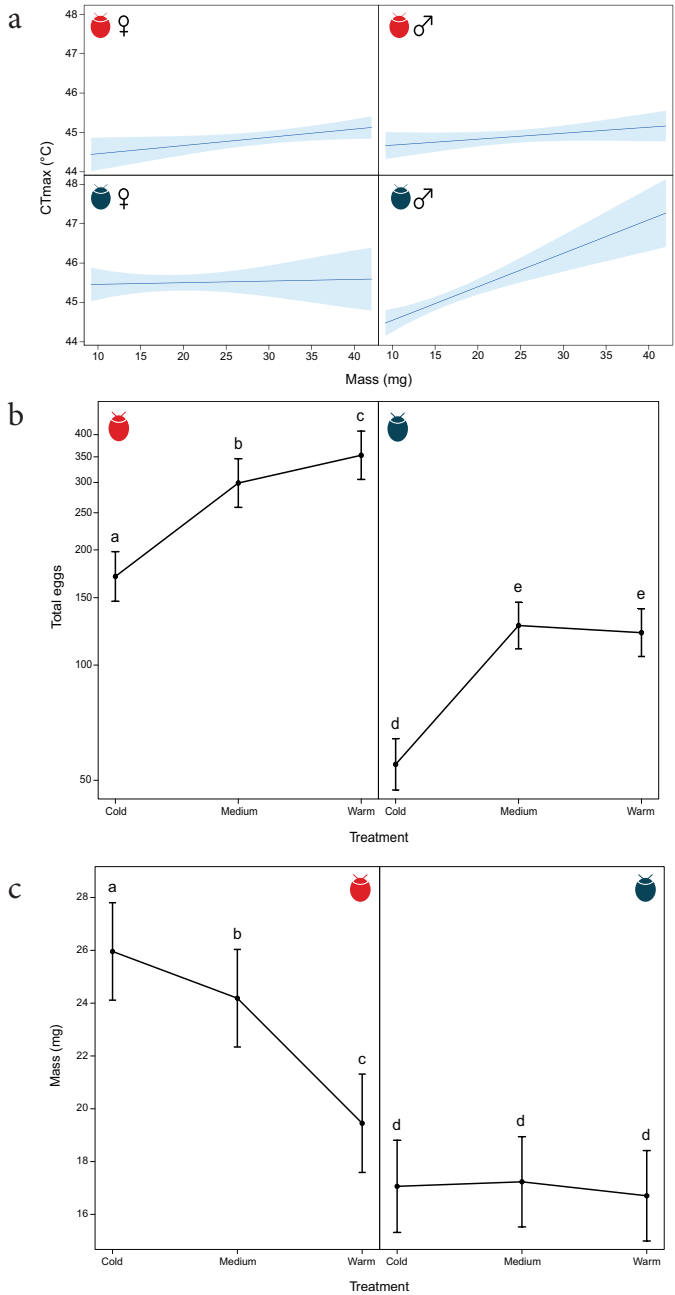


Figure 3. Contrasting responses of *Harmonia axyridis* and *Cheilomenes lunata* maintained at three temperature treatments. **a** critical thermal maxima (CT_{max}) of female and male *H. axyridis* (top row) and *C. lunata* (bottom row) as a function of body mass. Slopes are based on the best model parameter estimates **b** total number of eggs laid by *H. axyridis* (left) and *C. lunata* (right) at each temperature treatment **c** adult mass (mg) of *H. axyridis* (left) and *C. lunata* (right) in each treatment. In **b** and **c** different letters indicate significant differences between groups. Values are based on the best model parameter estimates and standard errors.

Table 1. Estimates of population size of *Cheilomenes lunata* and *Harmonia axyridis* across stages of invasion. Last column presents the number of individuals after a heatwave event in the novel site (see Suppl. material 1: fig. S9 for more details). Results include simulations in cold, medium and warm temperature regimes after 1000 repeats. *Cheilomenes lunata* is indicated by 'CL' and *Harmonia axyridis* 'HA'. Values are mean number of individuals \pm SD.

Species	Scenario	Adults surviving transport	Females surviving transport	Total eggs laid in novel site	Viable eggs in novel site
CL	Cold	26.7 \pm 26.6	13.6 \pm 13.3	815.2 \pm 813.4	747.9 \pm 745.3
CL	Medium	15.5 \pm 23.8	8.1 \pm 11.9	999.4 \pm 1469.5	877.1 \pm 1291.4
CL	Warm	9.0 \pm 19.0	4.9 \pm 9.4	671.0 \pm 1298.4	479.2 \pm 931.3
HA	Cold	20.7 \pm 28.2	10.7 \pm 14.0	1752.6 \pm 2311.6	1510.7 \pm 1993.9
HA	Medium	14.1 \pm 23.5	7.4 \pm 11.7	2401.0 \pm 3800.1	2176.2 \pm 3442.5
HA	Warm	10.6 \pm 21.6	5.7 \pm 10.7	2219.5 \pm 4191.7	1831.1 \pm 3467.1
Species	Scenario	Larvae in novel site	Adult offspring in novel site	Adults after a heatwave event	
CL	Cold	705.6 \pm 702.9	514.9 \pm 516.0	287.5 \pm 288.2	
CL	Medium	800.1 \pm 1177.7	596.0 \pm 875.7	364.4 \pm 535.1	
CL	Warm	418.2 \pm 810.9	322.0 \pm 625.1	273.1 \pm 529.7	
HA	Cold	1364.2 \pm 1799.2	1298.4 \pm 1712.1	225.0 \pm 297.3	
HA	Medium	2024.2 \pm 3201.8	1887.3 \pm 2982.2	502.2 \pm 794.2	
HA	Warm	1606.6 \pm 3041.3	1320.7 \pm 2497.7	669.0 \pm 1264.4	

between the cold and medium, and between the cold and warm treatments than the native species (species \times treatment interaction; Suppl. material 1: fig. S5). Overall, males had a faster developmental time than females ($p < 0.001$). Larger adults were associated with slower developmental times and this relationship slightly differed across treatments (mass \times treatment interaction; Suppl. material 1: fig. S6). *Harmonia axyridis* had a higher percentage of successful pupal emergence than *C. lunata* ($90.4 \pm 10.1\%$ ($n = 40$) and $75.6 \pm 21.7\%$ ($n = 37$), respectively; $p = 0.02$), but there were no differences between temperature treatments.

The mass of adults of *C. lunata* remained constant across treatments ($p > 0.28$ for all comparisons), while that of *H. axyridis* decreased with the treatment temperature ($p < 0.0001$ for all comparisons; species \times treatment interaction; Fig. 3c). Similar patterns emerged when examining sexes separately (sex \times species \times treatment interaction; Suppl. material 1: fig. S7). For both species, and across all treatments, males were smaller than females ($p < 0.001$ for all comparisons). *Harmonia axyridis* males and females were heavier than *C. lunata* males and females in the cold and medium treatments ($p < 0.001$ for all comparisons), but not in the warm treatment ($p > 0.14$ for all comparisons) (Suppl. material 1: fig. S7).

Harmonia axyridis had a higher intrinsic rate of increase (i.e. per capita change in the population per generation) than *C. lunata* overall (0.10 ± 0.03 ($n = 40$) and 0.09 ± 0.04 ($n = 37$), respectively; $p = 0.02$), but there was no significant effect of treatment (see Suppl. material 1: table S5 for means \pm SD and sample sizes for the net reproductive rate (R_0) and generation time (T_g) of both species for each temperature treatment). Larger reproductive females of *H. axyridis* had a higher intrinsic rate than smaller ones, while this relationship was not found for *C. lunata* (female mass \times species interaction; Suppl. material 1: fig. S8).

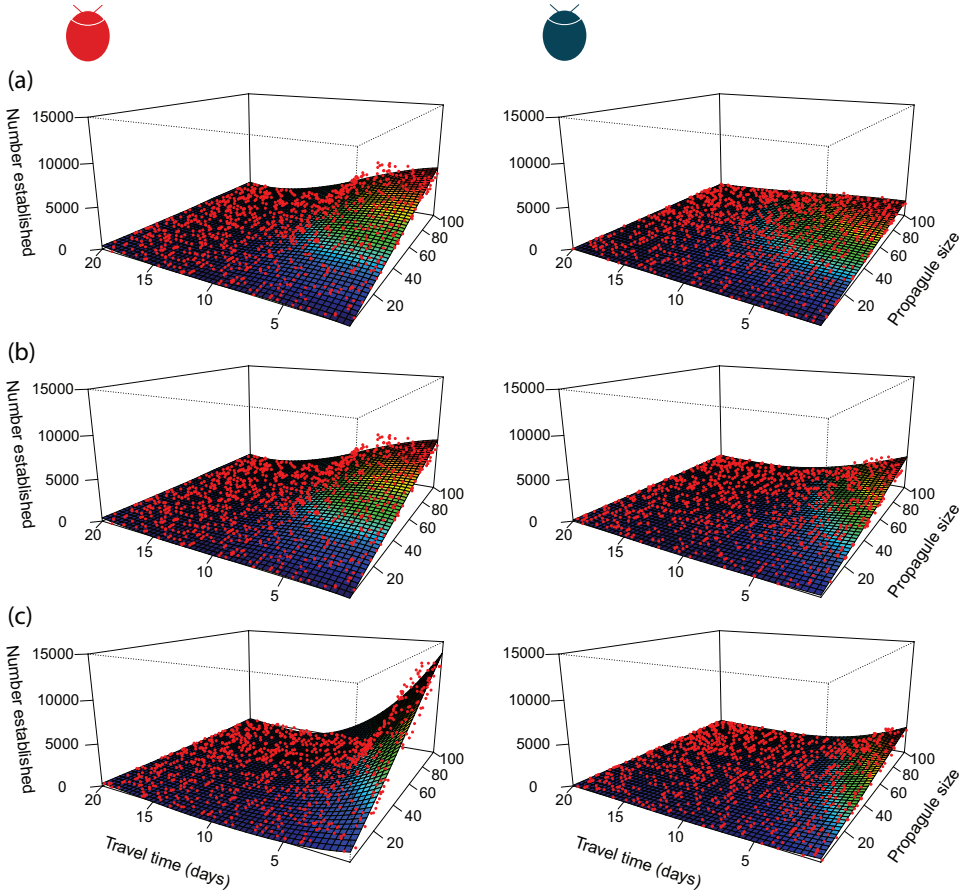


Figure 4. Predicted number of beetles established as a function of propagule size and traveling time. Plots present the number of individuals of *Harmonia axyridis* (left column) and *Cheilomenes lunata* (right column) that would survive the transport and establishment stages in **a** cold **b** medium and **c** warm environments (see assumptions, and starvation and fitness data collection in text). One thousand repeats were used and starting propagule size (1 to 100) and travel time (1 to 20 days) were randomly selected for each run. Outcomes of the iterations are shown as red points. Surface regression planes were obtained using locally estimated scatterplot smoothi.

Modelling population size

Once individuals survived transport and successfully arrived in the new area, more offspring of *H. axyridis* established than *C. lunata* in all temperature scenarios (Table 1). The average established population size (number of adult offspring) was highest in the medium scenario for both species (Table 1). In warm environments, high propagule pressure resulted in high levels of establishment in *H. axyridis*, while this was less notable for *C. lunata* (Fig. 4).

Discussion

This study showed that the native *C. lunata* had a greater upper temperature tolerance than the invasive *H. axyridis*, but *H. axyridis* had a higher performance than *C. lunata* for several life-history traits, in particular, fecundity and intrinsic rate of population increase. Despite the native species being more plastic for some traits (e.g. starvation resistance), *H. axyridis* displayed consistently higher performance over the range of temperature conditions compared to the native species (preoviposition period, number of eggs, % pupal emergence), fitting the model of a general purpose or Jack of-all-trades phenotype (Richard et al. 2006). The combination of traits, such as those linked to fitness, resulted in a consistently higher intrinsic rate of increase of the invasive species compared to the native one.

Studies that examine the plasticity of starvation resistance in invasive insects have found no thermal acclimation effects (e.g. Weldon et al. 2018), or that starvation resistance increases or decreases with temperature (e.g. Hoffmann et al. 2005). Knapp and Řeřicha (2020) exposed laboratory-reared introduced populations of *H. axyridis* to several months of cold, average and warm over-wintering temperature regimes and then scored starvation resistance at 22 °C. They found that after the cold winter-regime, beetles had reduced body mass loss and increased starvation resistance. However, the physiological mechanisms underlying starvation resistance after quiescence or winter diapause are likely to be very distinct from those of an acute food shortage as in our current study. We found that both *H. axyridis* and *C. lunata* survived longer without food in colder conditions, but *C. lunata* was more plastic for this trait, counter to the expectation of higher plasticity in the invasive species. This finding was not due to species' differences in the percentage of body mass loss as the native species lost a higher percentage of body mass than the invasive (Suppl. material 1: table S5), and there was a lack of a significant interaction between body mass lost, treatment and species explaining starvation resistance. Increased plasticity in the native species could result from an increased ability to store energy reserves, a reduction in their usage, or a higher tolerance for low body energy content (Rion and Kawecki 2007). However, we cannot confirm that the extent of food shortage stress imposed in our experiments was equivalent for both species due to unknown aspects that influence starvation resistance, especially for the native species (e.g. life span). Moreover, differences between sexes in starvation resistance and mass loss across temperature treatments for both species could be explained by different energy use and energy storage strategies, and their sensitivity to temperature change (Dmitriew et al. 2009; Hodek et al. 2012; Knapp and Nedvěď 2013; Knapp 2014). Since starvation resistance impacts dispersal ability, females may be better dispersers than males in cold and optimal environments, but males may cope better in warm environments.

Thermal tolerance limits of insects are often plastic and thermal acclimation effects are typically more notable for CT_{min} than CT_{max} (e.g. Terblanche et al. 2010; Lancaster et al. 2015; Weldon et al. 2018). A few studies have also reported increased plasticity or faster time course of acclimation in invasive compared to native species (e.g. Kellet

et al. 2005; Nyamukondiwa et al. 2010; Mutamiswa et al. 2018). Our results are in line with thermal acclimation of temperature limits in insects. For both species, acclimation to colder temperatures resulted in lower CT_{min} . However, for CT_{max} , complex interactions with sex and mass differentiated the species. Overall, basal upper tolerance was slightly higher in the native species, and these differences, albeit their small effect size, can elicit negative effects on survival when facing extreme warm events. For example, by simulating a stochastic heatwave event for established beetles of either species in the form of a temperature exposure to 45 °C, we find that the percentage mortality of *H. axyridis* is higher than *C. lunata* in all temperature scenarios (Cold: 83% vs 44%, Medium: 73% vs. 39%, Warm: 49% vs 15% mortality for *H. axyridis* and *C. lunata*, respectively; see Suppl. material 1: fig. S9). Despite this high mortality however, the population size of *H. axyridis* would remain larger than the native species even when both species are transported and introduced with the same propagule size, except for the cold scenario (Table 1). This simple simulation suggests that predictions of global warming effects on successful invasive species and native species require examining performance beyond the plasticity of critical limits alone and need to incorporate simulations of population fluctuations that are mediated by other factors (e.g. initial propagule size), traits (life-history) and associated adaptive processes (e.g. plasticity and evolutionary potential of thermal traits; Garnas 2018; Logan et al 2019). While this may seem obvious, the direction and consequences of these effects on population size can often be counterintuitive (Sgrò et al. 2016) as for example, plastic effects such as those reported here, can be of small magnitude.

Given the cold origin of *H. axyridis*, and its recent introduction to South Africa (early 2000s; Stals 2010; Roy et al. 2016), the native *C. lunata* may be better adapted to local conditions compared to *H. axyridis*. However, exploring the biogeography and invasion history of *H. axyridis* in South Africa, and the microclimates used by both species in the field, should be paramount to address this question, as environmental heterogeneity and associated costs and risks of using the environment could underlie plastic responses in these species (Hoffmann and Sgrò 2018; Shinner et al. 2020).

Previous studies on life-history traits have found mixed results regarding consistent plastic responses of traits in invasive insects as fecundity, offspring survival and developmental time to temperature (e.g. Kingsolver et al. 2007; Terblanche et al. 2010; Ferrer et al. 2014; Fält-Nardmann et al. 2018). Invasive *H. axyridis* performed better over a broader range of conditions than *C. lunata* for the majority of life-history traits (with the exception of hatching success and developmental time) but we found no notable differences in plasticity of these traits between species. A meta-analysis that compared life-history traits of *H. axyridis* in its introduced range (i.e. Europe and North America) and native range showed that invasive beetles had higher mean fecundity (Raak-van den Berg et al. 2017), perhaps highlighting the role of this trait in driving the invasiveness of *H. axyridis*. Several studies reported that its fecundity was highest at intermediate temperatures (24–27 °C) compared to colder (18–20 °C) and warmer (30 °C) temperatures (Lombaert et al. 2008; de Oliveira Ramos et al. 2014; Barahona-Segovia et al. 2016). In our study, fecundity increased consistently with temperature

(Fig. 3b), but the warm regime (fluctuations between day-time $\sim 30^{\circ}\text{C}$ and night-time $\sim 23^{\circ}\text{C}$) was likely more benign compared to the typical chronic exposures used in these other studies. Fluctuating temperatures may increase performance as the overall temperature regime remains within the permissive range, and night-time temperatures can allow repair of potential damage incurred during daytime (Colinet et al. 2015).

Combining species' traits and their plasticity in an invasion framework demonstrated that, given the same variation in propagule pressure and stages of invasion, a larger number of invasive beetles will establish compared to the native, but medium temperature conditions will maintain the highest numbers for both (Table 1). Several patterns emerged when assessing how species' traits collectively affected population size at the novel site, depending on the temperature scenario. First, in all temperature scenarios, travel or transportation time drastically reduced establishment, especially at warm temperatures (Fig. 4). Second, for both species, at least 30 propagules were required at all temperatures for a few individuals to survive transportation and establish, but the patterns that emerged from the transport stage onwards deviated substantially between species. Third, both species were able to resist starvation for longer during transport in the colder scenario, but a greater number of eggs laid in the medium scenario meant that both species were able to compensate for losses incurred during transport and establish the highest number of individuals. Simulations showed that, despite the constraining effects of lower starvation resistance at warm temperatures, high numbers of *H. axyridis* were able to establish given the high fecundity at the warm regime and a sufficiently large propagule size. By contrast, for the native *C. lunata*, the number of individuals that established in the warm scenario was compromised due to the low number of individuals that survived the transport stage coupled with reduced fitness.

Our results should be interpreted with caution for several reasons. While there is a fair amount of information about *H. axyridis* thermal biology and life-history, we have less knowledge of the native species' general biology and limited understanding of microclimates experienced by coccinellids in general (Roy et al. 2016; Sloggett et al. 2021). Therefore, the temperature regimes and protocols used may be more ecologically relevant to one of the species; nevertheless, from our own observations, both species frequently co-occur on the same plant microsites and are observed feeding on the same aphid prey. In addition, differences in the shape of performance curves between species combined with the fluctuating environmental conditions could result in different realized fitness outcomes (Colinet et al. 2015; Denny 2017). This is not problematic if the fluctuating regimes used here are relevant to both species. Finally, more facets of these species' ecology are needed to refine the predictions on their colonization and establishment potential in new thermal environments. Plasticity in phenological, behavioral and developmental responses that allow persistence or avoidance of stressful conditions, as well as dispersal and competitive abilities, would be essential (Hulme 2008; Turcotte and Levine 2016). For example, although both species are primarily aphidophagous, *H. axyridis* can be polyphagous and cannibalistic during periods of its life cycle (Wagner et al. 1999; Snyder et al. 2000). These characteristics are unknown for the native species.

Regardless of these potential limitations, we show that for a set of key traits, the invasive species had higher performance than a co-occurring native species, mostly resulting from higher mean effects and despite both species having some plasticity for different traits. This study demonstrates that making interpretations from a reduced set of performance traits or invasion stages would present an inaccurate estimation of these species' potential establishment into new thermally-distinct areas. Baseline knowledge of traits, plus their variability in different thermal environments as examined here, is essential if we aim to predict the response of successful invasive and native species to future climate scenarios. Disentangling between 'active' and 'passive' thermal plasticity (Havird et al. 2019) will be a useful next research focus to improve predictions of these species' responses to unprecedented temperature variations, both acute and long-term changes, associated to climate change.

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Supplementary material I

Tables and figures

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Data type: Docx file.

Explanation note: **Tables: S1.** Incubator temperatures (IT in °C) at which *Harmonia axyridis* performed best in terms of reproduction and population growth characteristics. **S2.** *Harmonia axyridis* and *Cheilomenes lunata* collection sites (GPS coordinates) in Stellenbosch (Western Cape Province, South Africa). **S3.** Best model outputs for each trait: (a) Starvation resistance, (b) CTmin, (c) CTmax, (d) preoviposition period, (e) total eggs laid, (f) hatching success, (g) developmental time, (h) pupal emergence success, (i) adult mass, and (j) intrinsic rate of increase. **S5.** Summary statistics (mean, standard deviation and sample size) for each trait and temperature treatment: (a) starvation resistance, (b) thermal tolerance, (c) life-history traits. **S6.** Averaged model summary outputs: (a) CTmin, (b) CTmax, (c) developmental time, and (d) hatching success. HA = *Harmonia axyridis*, CL: *Cheilomenes lunata*. **Figures: S1.** Study experimental design for rearing and determining physiological and life-history traits of the two beetle species. T1 to 3: treatments 1 to 3. CTL = Critical Thermal Limits. Ladybird illustration by Corneile Minnaar. **S2.** Kaplan-Meier survival curves used to plot starvation resistance data of *Harmonia axyridis* and *Cheilomenes lunata* for the cold (right - blue), medium (middle - green) and warm (left - orange) temperature treatments for beetles that had a) low mass loss ($\leq 16.43\%$, the median mass loss percentage for all beetles in all treatments) and b) high mass loss ($> 16.43\%$) groups. **S3.** Critical thermal maximum (CTmax, °C) of male and female beetles of *Harmonia axyridis* and *Cheilomenes lunata* for each temperature treatment. **S4.** Values are model parameter estimates. Total number of eggs laid by *Harmonia axyridis* (top row) and *Cheilomenes lunata*. **S5.** Developmental time (days) from egg to pupal emergence of *Cheilomenes lunata* (left) and *Harmonia axyridis* (right) in each temperature treatment. Values are model parameter estimates. **S6.** Developmental time (days) of *Harmonia axyridis* and *Cheilomenes lunata*. **S7.** Adult (F3) mass (mg) of male and female *Harmonia axyridis* (top row) and *Cheilomenes lunata*. **S8.** Intrinsic rate of increase of *Cheilomenes lunata* (left) and for *Harmonia axyridis* (right) as a function of female mass (mg). **S9.** Density plots of measured critical thermal maximum (CTmax) data for *Harmonia axyridis*.

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