RESEARCH ARTICLE



Comparing environmental impacts of alien plants, insects and pathogens in protected riparian forests

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Abstract

The prioritization of alien species according to the magnitude of their environmental impacts has become increasingly important for the management of invasive alien species. In this study, we applied the Environmental Impact Classification of Alien Taxa (EICAT) to classify alien taxa from three different taxonomic groups to facilitate the prioritisation of management actions for the threatened riparian forests of the Mura-Drava-Danube Biosphere Reserve, South East Europe. With local experts we collated a list of 198 alien species (115 plants, 45 insects, and 38 fungi) with populations reported in southeast European forest ecosystems and included them in the EICAT. We found impact reports for 114 species. Eleven of these species caused local extinctions of a native species, 35 led to a population decrease, 51 to a reduction in performance in at least one native species and for 17 alien species no effects on individual fitness of native

species were detected. Fungi had significantly highest impact and were more likely to have information on their impacts reported. Competition and parasitism were the most important impact mechanisms of alien species. This study is, to our knowledge, the first application of EICAT to all known alien species of several taxonomic groups in a protected area. The impact rankings enabled to identify taxa that generally cause high impacts and to prioritize species for the management in protected areas according to their impact magnitudes. By following a standardized impact protocol, we identified several alien species causing high impacts that do not appear on any expert-based risk list, which are relevant for policymakers. Thus, we recommend that alien species be systematically screened to identify knowledge gaps and prioritize their management with respect to spatio-temporal trends in impact magnitudes.

Keywords

Alien species, biological invasions, EICAT, invasive species management, protected areas, species prioritization

Introduction

Invasive alien species are a major threat to European forest ecosystems (CBD 2001; FAO 2009; Europe and Unece 2015). Globally, they have become the second most common extinction threat to endangered species due to the increasing human-mediated transportation of species far beyond their native range (Bellard et al. 2016). Previous studies on individual or multiple alien species have revealed severe impacts of alien species on ecosystem functions, ecosystem services, and biodiversity in forest ecosystems (Seidl et al. 2018); these impacts are linked to a multitude of impact mechanisms: parasitism, competition with native species, physical changes to the environment, and pathogen transfer (Kenis and Branco 2010; Pyšek et al. 2012; Ricciardi et al. 2013; Langmaier and Lapin 2020).

As a result of the rapidly increasing impact of biological invasions, the control of invasive alien species – i.e. any species or lower taxon of animals, plants, fungi, and other microorganisms whose occurrence in a region outside its natural range that has negative impacts on an ecosystem and its services (CBD 2002) – has been implemented in international, national, and regional policies and legislations such as the EU Biodiversity Strategy or EU Regulation No. 1143/2014 on invasive alien species. Their aim is to mitigate the ecological and socioeconomic effects of alien species. The few cross-taxon assessments performed have shown that terrestrial invertebrates, and terrestrial plants in particular, are associated with ecological and economic impacts in Europe (Vilà et al. 2010; Kumschick et al. 2015).

Riparian forests are highly vulnerable to biological invasion (Marinšek and Kutnar 2017; Medvecká et al. 2018). Their high nutrient levels and frequent natural and man-made disturbances facilitate invasions, and the rivers themselves serve as effective corridors for the spread of alien species (Kowarik 1992; Pyšek and Prach 1993; Schmiedel et al. 2013; Lapin et al. 2019). Management of alien species in riparian areas is therefore essential for preserving and restoring the biodiversity and ecosystem services of these endangered ecosystems (Rivers et al. 2019). However, the resources for conservation management in protected riparian forests are limited and require effective prioritization. A cross-taxon impact assessment, of the alien species present or likely to be present in the near future, because the species have been observed in neighboring areas, in a protected area could be useful for the prioritization of management actions and to facilitate the evaluation of management methods (Roy et al. 2019; IUCN 2020b).

Besides horizon scanning frameworks (Roy et al. 2019) and risk assessment protocols, scoring systems for impact assessments have thus gained considerable importance not only for policy makers or the scientific community, but also for conservation managers of protected areas. Several tools have been developed to quantify, compare, and prioritize the impact of alien species (Vilà et al. 2019). The generic impact scoring system (GISS), for example, focuses on the environmental and socio-economic impacts of alien species (Nentwig, et al. 2016). Here, we follow the scoring system of the Environmental Impact Classification of Alien Taxa (EICAT), which classifies alien taxa in terms of the magnitude of their highest observed environmental impacts in recipient areas, based on the level of organisation impacted of a native species and its reversibility (Blackburn et al. 2014; Hawkins et al. 2015). Recently, the International Union for Conservation of Nature adopted EICAT as a global standard similar to the IUCN Red List for extinction threat (IUCN 2020d).

In the past few years, EICAT has been widely applied and discussed (Kumschick et al. 2017; Kumschick et al. 2020). However, most impact assessments have primarily focused on EICAT classification within single taxonomic groups, such as global impact assessments of birds (Evans et al. 2016), ungulates (Volery et al. 2021), bamboos (Canavan et al. 2019), or amphibians (Kumschick et al. 2017), while only few studies have performed cross-taxon assessments. Even fewer studies have undertaken cross-taxon assessments for a specific habitat or a geographic region (Shivambu et al. 2020). This study investigates the cross-taxon impacts of alien species in order to facilitate the prioritization of management actions for the endangered riparian forests of the transboundary UNESCO Mura-Drava-Danube Biosphere Reserve in Southeast Europe. The riparian forest of the Biosphere Reserve was selected as a representative protected area for the European challenge to combat the spread of invasive alien species.

The objectives of the study are (1) to provide a cross-taxon impact assessment of alien taxa, in the Mura-Drava-Danube Biosphere Reserve, in terms of the magnitude of their highest observed environmental impacts in riparian temperate forests in Europe, (2) to determine differences in the impact severity and impact mechanisms of fungi, insects, and plants, with consideration for the time period since their introduction (residence time), (3) to identify knowledge gaps and the availability of data on alien taxa for application of the cross-taxon impact assessment. With our work we wish to support the prioritization of taxa for control and management within this vulnerable riparian ecosystem. Additionally, we quantify environmental impacts on forest ecosystems, thereby supporting forest management decisions.

Methods

Area description

The Mura-Drava-Danube Biosphere Reserve covers an area of nearly 850,000 ha in the countries of Austria, Slovenia, Hungary, Croatia and Serbia. The entire core zone of this important ecological corridor - a belt of riparian forests along the three rivers - has been designated as part of the Natura 2000 framework and contains protected areas of various categories. New parts of the Biosphere Reserve were recently nominated and now it is the largest protected river area in Europe and the only UNESCO Biosphere Reserve spanning across five countries. A share of 27% of the Biosphere Reserve is covered by forest. This portion increases to 61% within the core zone. Between the countries, there are remarkable differences regarding the ownership structure and forest management practices. The annual mean temperature ranges from 9.3 °C in the north-western part of the study area to 11.7 °C in the area between Đurđevac (Croatia) and Barcs (Hungary). The whole Biosphere Reserve shows strong variation of annual precipitation ranging from sites with nearly 1000 mm in the West to almost 500 mm in the North-Eastern Hungarian part of the Biosphere Reserve. The Biosphere Reserve is characterized by highly fertile plains along the rivers with an intense agricultural use for cereal, maize and pasture cropping on the one hand, and forestry on the other. The rivers are embedded in eutric Fluvisols (33%), surrounded by Luvisols (14%) and Cambisols (5%). Phaeozems (35%) are the dominant soil type.

Data collection

A list of 390 alien species (165 fungal species – including species of pseudo-fungi, 48 insect species, and 177 plant species) with reported populations in Southeast European forest ecosystems was extracted from the Global Invasive Species Compendium database using the invasive species Horizon Scanning Tool (beta) (incorporating data up to March 2019, (CABI 2018). Additional information on alien species from the observations of Austrian, Slovenian, Croatian, Serbian, and Hungarian national experts and the alien species alert and observation list from the "Life Artemis project" (DeGroot et al. 2017; Marinšek and Kutnar 2017) was included. In total, 188 alien species were excluded by the expert panel of assessors before the beginning of the assessment process because these species do not generally occur in riparian forest ecosystems and exhibit a very low potential occurrence in the riparian forests of the Biosphere Reserve. Ultimately, 198 species (115 plants, 45 insects, and 38 fungi) were included in the list of alien species (Appendices 1, 2).

The 198 species were distributed among the assessors. All assessors and reviewers were invited to a workshop in September 2019 during which the EICAT assessment protocol was demonstrated and practiced. The assessors had different backgrounds and years of expertise, e.g. geneticists, biodiversity conservationists, forest science and also junior staff/technicians. The applied assessment protocol followed the Guidelines for

using the IUCN Environmental Impact Classification for Alien Taxa (EICAT) Categories and Criteria (IUCN 2020b, c; Volery et al. 2020). The assessors undertook a review of published literature and local reports to identify the environmental impact of the selected 198 alien species in forests. The databases Google Scholar and Scopus were used along with Google web searches to collate publications. We adapted the EICAT protocol search string in order to focus only on impacts observed in forest ecosystems using the following search terms: "forest" AND "Europe" AND ("introduced species" OR "invasive species" OR "invasive alien species" OR "IAS" OR "alien" OR "non-native" OR "non-indigenous" OR "invasive" OR "pest" OR "feral" OR "exotic"). Publications describing an environmental impact in a different ecosystem type or other climatic regions than temperate climate were not included. Each record was assessed separately. The impacts identified in the literature were classified according to their magnitude following five categories: minimal concern (MC), minor (MN), moderate (MO), major (MR) or massive (MV). Following the EICAT protocol, each alien taxon was assigned an EICAT category based on its highest observed impact across all recorded impacts. The impact mechanisms for each alien species were also identified from the assessed publications and categorized into one of 12 impact mechanism categories as defined in the EICAT guidelines (IUCN 2020b, c; Volery et al. 2020). Insect herbivory was included in the impact mechanism 'Parasitism', because these insects are not killing but parasitizing on the trees. All assessments were independently crossvalidated for consistency by an assigned independent reviewer in three review loops. The final scores were agreed upon by consensus among all authors, which was reached in constructive discussions in several online-meetings.

Data analysis

Microsoft Excel 2010 was used for the data management, and R version 3.4.2 (R Core Team 2017), with the libraries "ordinal" (Christensen 2019), "stats" (R Core Team 2017) and "ggplot2" (Villanuev et al. 2016) for data analysis together with Python version 3.7 (Van Rossum and Drake 2009). For analysis of the respective alien species' native region, we categorized the area of geographic origin by continents (Africa, Asia, Australia, Europe, North (including Central) America, and South America). The time of the first record in the wild in Europe was included to analyze the influence of residence time on a species' impact. This information was obtained by reviewing scientific literature on the first records of each species.

We calculated the concurrence (Con) to analyze whether obtained EICAT impact categories vary among impact reports as well as the variance in impact magnitudes (Var) of the impact reports of each alien taxon regarding their impact categories across the impact mechanisms and taxonomic groups. For the analysis of both, the concurrence and variance, only alien species with two or more assessed impact reports were included. In total, 59 species with multiple impact reports per alien species were analyzed regarding their dissimilarity in the consensus on the impact category. For the concurrence we used the percentage of references within the most frequent category (the category with the most references assigned to the species assessments). In the next step, we calculated the average percentage for a) each mechanism and b) each taxonomic group individually. The calculation of concurrence implied the division of the number of references of the most frequent impact category (n_{freq}^i) by the total number of references (n_{total}^i) within the same species i, which was performed for each species individually. We then calculated the sum of all individual species by mechanisms, respectively taxonomic groups. To arrive at concurrence, we divided the resulting sum by the number of species (N) for each mechanism respectively for each taxonomic group. In this result, a high percentage indicates high consensus whereas a low percentage indicates low consensus. The equation for concurrence is as follows:

concurrence =
$$\frac{1}{N} * \sum_{i=0}^{M} : \cdots : \frac{n_{\text{freq}}^{i}}{n_{\text{total}}^{i}} * 100$$

For the variance in impact magnitudes, we investigated the statistical variance of the different EICAT impact categories, calculating the average percentage for a) each mechanism and b) each taxonomic group individually. A high variance score indicates high dissent.

We modelled the effect of the explanatory variables taxonomic group, geographic origin (southern or northern hemisphere), and years since first record in the wild in Europe on the maximum EICAT impact category per species. As the response variable of impact categories was ordinal, we used cumulative link models (CLM). For the model selection, the Akaike Information Criterion (AIC) was used in which all models within 2 AIC units from the lowest AIC were chosen as the best models (Anderson and Burnham 2002).

The residence time was analyzed for the difference with taxonomic group and impact category. An ANOVA was used between residence time compared to taxonomic group, impact category and their interaction. With the model selection, all models within 2 AIC units from the lowest AIC were chosen as the best models.

For analyzing the data deficiency of the impacts per species, we used a generalized linear model (GLM) with binomial error structure. The dependent variable was based on the presence and absence of an impact description. The independent variables were taxonomic groups, years since the first recorded introduction to Europe and geographic origin. We used a backward stepwise model selection to come to the best model on the basis of the AIC (Burnham and Anderson 2002). All models within 2 AIC units from the lowest AIC were conditional average.

Results

In total, 303 references with information on 114 alien species were used, with an average of 2.7 ± 0.14 (mean \pm SE) references per species. The average number of references for plants was 2.8 ± 0.06 and thus lower than the average of 3.2 ± 0.06 for insects but

| Taxonomic | Species | Impact mechanism | Origin | Years of introduction |
|-----------|----------------------------|----------------------------------|---------------|-----------------------|
| group | | | | to Europe |
| Fungi | Biscogniauxia mediterranea | (5) Parasitism | North America | 1931 |
| | Botryosphaeria dothidea | (5) Parasitism | Europe | - |
| | Cryphonectria parasitica | (5) Parasitism | Asia | 1938 |
| | Hymenoscyphus fraxineus | (1) Competition | Asia | 1990 |
| | Ophiostoma novo-ulmi | (5) Parasitism | Asia | 1990 |
| Plants | Amorpha fruticosa | (1) Competition | North America | 1724 |
| | Heracleum persicum | (1) Competition | Asia | 1817 |
| | Humulus japonicus | (1) Competition | Asia | 1880 |
| | Impatiens glandulifera | (1) Competition | Asia | 1839 |
| | Reynoutria japonica | (9) Chemical impact on ecosystem | Asia | 1851 |
| | Reynoutria sachalinensis | (1) Competition | Asia | 1860 |

Table 1. Results of the EICAT assessments indicating species that have caused on at least one occasion a local extinction of a native species and thus are listed in the most harmful impact category assessed in this study: MR (Major) (IUCN 2020b).

higher than the average number of species references for fungi which was 1.89 ± 0.05 . It is important to note that for most species only one single reference was available, as the mode for all individual taxonomic groups was equal to 1. The references used extended across a time span of 39 years, with the oldest one published in 1981 and the most recent one in 2020. The results show that, in total, 11 alien species (Plants: n = 6, Fungi: n = 5) were assessed as having caused on at least one occasion a Major impact, which led to the naturally reversible local extinction of a native taxon (i.e. change in community structure). A Major impact was the most harmful impact category of the 114 alien species assessed (Table 1); No alien species were assigned to the highest and most harmful impact category Massive (naturally irreversible local or global extinction of a native taxon). 35 alien species were assigned to the impact categories Moderate and caused population decline, 51 to Minor and caused reduction in individual performance and 17 to Minimal Concern and had no or negligible impact on other native species, across the taxonomic groups – plants, insects, and fungi, as shown in Figure 1. The full list of EICAT assessment results is provided in the Appendix 1: Table A1.

Most of the assessed alien species originate from North America (56.1%), followed by Asia (36.0%), Australia (1.3%), South America (0.69%), Africa (0.6%), and 3.0% were native in Europe, but non-native to the study area. The distribution of impact categories differed between taxonomic groups as well as in terms of years elapsed since the first introduction to Europe, i.e. residence time (Figure 1). Residence time was only different between taxonomic groups (LR Chisq = 95.52, df = 2, P < 0.001). Plants exhibited the longest residence time (years since the first recorded introduction to Europe), while fungi and insects were recorded to arrive in Europe more recently (Figure 2).

We classified nine different impact mechanisms for 114 alien species, through which environmental impacts were caused (Table 2). Overall, the most frequent impact mechanisms were Parasitism (49 alien species, or 43.0%), Competition (29 alien species, or 25.4%), and Structural impact on ecosystems (8 alien species, or 7.0%).



Figure 1. Relative frequency of EICAT impact categories (total species = 114) across the taxonomic groups of insects (n = 25), plants (n = 55) and fungi (n = 34).



Figure 2. Box plots of the residence time in Europe (years since first report) for species in different taxonomic groups and impact categories: Major (MR), Moderate (MO), Minor (MN), and Minimal Concern (MC).

| Taxonomic group | Impact mechanism | concurrence | Variance | Number of references |
|-----------------|------------------------------|-------------|----------|----------------------|
| Fungi | Competition | 75.00 | 1.00 | 4 |
| | Parasitism | 80.90 | 0.23 | 32 |
| Insects | Parasitism | 90.38 | 0.17 | 24 |
| Plants | Chemical impact on ecosystem | 83.33 | 0.67 | 4 |
| | Competition | 66.28 | 0.42 | 34 |
| | Hybridization | 50.00 | 2.00 | 2 |
| | Indirect impacts | 62.50 | 1.03 | 5 |
| | Parasitism | 76.67 | 0.53 | 14 |
| | Physical impact on ecosystem | 62.50 | 0.38 | 3 |
| | Poisoning / Toxicity | 100.00 | 0.00 | 4 |

Table 2. Results of the concurrence and variance of the impact categories across the impact mechanisms and taxonomic groups.



Figure 3. Distribution of the assessments by taxonomic group; the x-axis represents the impact categories: Major (MR), Moderate (MO), Minor (MN), Minimal Concern (MC); the y-axis shows the number of references in the respective category (bars).

This order varied among the different taxonomic groups: For fungi the most frequent impact mechanism was found to be Parasitism (87%) followed by Competition (11%) and, lastly, Hybridisation (1%). For insects, Parasitism occurs most frequently (90%), followed by Structural impact on the ecosystem (6%) and Predation (2%). Whereas for plants Competition (50%) occurred more frequently followed by Parasitism (22%) and Structural impact on the ecosystem (9%).

The impact category with the most references found was Moderate (MO) for plants, and Minor (MN) for fungi and insects (Figure 3). Furthermore, we identified differences in the variability of impact magnitudes (concurrence) across taxonomic groups (Appendix 2: Table A2): Assessments of alien species from the taxonomic group insects varied the most (highest concurrence 87.5%, SD = 0.1), followed by fungi (concurrence = 82.2%, SD = 2.9), and plants (concurrence = 65.9%, SD = 15.2). The consensus concurrence on impact categories across impact mechanisms was the lowest for Competition (concurrence = 66.6%, SD = 4.3) and the highest for Transmission of diseases (concurrence = 100%, SD = 0.0) (Table 2).

The best model explaining the impacts of the invasive alien species included explanatory variables taxonomic group and geographic origin (Hemisphere) (Table 3).

Table 3. Results from the cumulative link model (CLM) demonstrating the relationship between the impact category of the EICAT impact assessments and explanatory variables: taxonomic groups and native geographic origin, showing the parameter estimates for the minimum adequate CLM; * P < 0.05, ** P < 0.01. The taxonomic groups were compared to plants and the southern hemisphere is compared to the northern hemisphere. The estimate shows the slope or the estimated difference from the reference level.

| Variables | Estimate | Std. error | z value | $\Pr(z)$ | |
|------------------------|----------|------------|---------|------------|----|
| Taxonomic group-insect | -1.773 | 0.547 | -3.244 | 0.001 | ** |
| Taxonomic group-plant | 0.048 | 0.448 | 0.107 | 0.914 | |
| Hemisphere-South | -1.663 | 0.917 | -1.813 | 0.07 | |

Table 4. Model statistics of the averaged model within 2 AIC units from the best model, explaining the influence of factors on the data deficiency of invasive alien species impact in the forests. * P < 0.05, ** P < 0.01. Estimate shows the slope or the estimated difference from the reference level.

| Variable | Estimate | Std. Error | z value | Pr (> z) | |
|--------------------------|----------|------------|---------|---------------------------|----|
| (Intercept) | -5.113 | 3.608 | 1.406 | 0.160 | |
| Taxonomic group-insect | -2.369 | 0.798 | 2.945 | 0.003 | ** |
| Taxonomic group-plant | -1.699 | 0.827 | 2.038 | 0.042 | * |
| Years since Introduction | 0.004 | 0.002 | 2.160 | 0.031 | * |
| Southern Hemisphere | -0.771 | 0.835 | 0.916 | 0.360 | |

The parameter estimates were provided by the likelihood confidence intervals. Insects had a significantly lower impact on native forests than fungi, while plants had a similar impact to fungi (Table 3). Alien species from the Southern hemisphere had a lower impact than species from the Northern hemisphere although the difference in impact was not significant (Table 3).

We were unable to conduct an EICAT impact assessment for 84 alien species due to data deficiency. For the data deficiency, the averaged model included the year of introduction, the taxonomic group and geographic origin (Table 4, Figure 4). The averaged model showed that for all taxonomic groups the impact descriptions were more likely to be found for the recently introduced species (Table 4). Furthermore, the fungi had a higher probability for an impact to be described than the insects and the plants (Table 4). There was no difference between alien species coming from both hemispheres in data deficiency.

Discussion

The management of harmful invasive alien species has become one of the greatest technical and financial challenges for the management of protected areas (Foxcroft et al. 2019; Mill et al. 2020). The prioritization of alien taxa is essential for setting costeffective management goals, for high priority species, which possess a severe negative impact. This is particularly important when a large pool of alien species is present (Campagnaro et al. 2018; Fogliata et al. 2021), like in the riparian forest of the UN-



Figure 4. The influence of time of the first record in the wild in Europe (x-axis) for **A** fungi **B** plants and **C** insects on the probability of an impact report of an alien species(y-axis). The dots show the actual presence and absence of impact reports and the line shows the prediction line of the model in Table 4.

ESCO Mura-Drava-Danube Biosphere Reserve. As with many other protected areas in Europe, a the Mura-Drava-Danube Biosphere Reserve also relies on transnational cooperation to face the common cross-border challenge adapting forest management to climate change, as well as for conservation of riparian forest ecosystems (Turnock 2002; Sallmannshofer et al. 2021). A prioritization of alien species is especially important to combat the spread of most harmful invasive alien species by harmonizing the management efforts of various administrations in the transboundary protected area.

Using the EICAT assessment, this study successfully categorized impacts on European forest ecosystems caused by 114 alien species of three taxonomic groups (plants, insects, and fungi) with reported populations in Southeast European forest ecosystems, all of which might pose a threat to the UNESCO Mura-Drava-Danube Biosphere Reserve. The information on environmental impacts was available for 90% of the fungi, 52% of the plants and 44% of the insects. The fact that more information was available for fungi is likely due to the small number of fungi included on the list of potentially occurring alien species in the assessment area (only 19% of 189 alien species were fungi). Moreover, although the tools and methods to identify fungal species have been positively influenced by advances in molecular biology, proper identification as well as invasion biology of fungi and fungal-like organisms have not yet been sufficiently explored. This is of particular importance as control measures depend on proper identification of diseases and their causal agents (Chetana et al. 2021). In addition, in this study we specifically assessed the impact of alien taxa on European forest ecosystems, which are highly affected by invasive alien species (Seidl et al. 2014). Therefore, impact reports were limited to observed impact on European forest ecosystems; well-described impacts on agriculture and horticulture (DiTommaso et al. 2016; Aneva et al. 2018) were not included in the assessment and are not covered in EICAT. This focus on impacts on forest ecosystems allowed us to provide a cross-taxon classification for the protected riparian forests of the Biosphere Reserve, as well as to identify reported impact mechanisms and knowledge gaps, and to facilitate discussions among local experts and stakeholders in the assessment area. Furthermore, our study shows that many invasive alien species are particularly affecting the riparian forest ecosystems. For instance, the fungi Hymenoscyphus fraxineus caused a population decline of the tree species Fraxinus excelsior, which is an important target tree species of the habitat type 91F0 (Riparian mixed forests of Quercus robur, Ulmus laevis and Ulmus minor, Fraxinus excelsior or Fraxinus angustifolia, along the great rivers (Ulmenion minoris)) under the EU Habitat directive. It has been shown that Fallopia spp. changes the chemistry of the litter layer and outcompetes the native species, this especially affects the herb layer but also the growth of the saplings, hence the reproduction of the riparian forests (Lavoie et al. 2018).

The assessment of the current impact information showed that none of the 114 alien species were categorized with the EICAT impact category Massive (MV), because the reported impacts unlikely result in irreversible extinctions of native species populations in the context of EICAT (IUCN 2020a). However, six alien plants and five alien fungi were found at the top of the ranking list of harmful alien species – classified in the EICAT category 'Major' (MR) – leading to local extinctions of native species in European forest ecosystems. For example, the Himalayan balsam (*Impatiens glandulifera* Royle) has been observed to have negative impacts on herbaceous native plant species diversity due to shading, which led to local extinctions (Čuda et al. 2017; Tanner and Gange 2020). The impacts of *I. glandulifera* are recognized across Europe and therefore this species is also included on the list of invasive alien species of Union concern (Regulation (EU) 1143/2014). In total, five alien plants (Major

impact: Impatiens glandulifera, Humulus scandens; Moderate impact: Heracleum mantegazzianum, Asclepias syriaca, Ailanthus altissima) in the upper ranking of this study are considered as invasive species on the Union List and therefore subject to restrictions and measures set out in the Regulation (EU) 1143/2014. Other alien species in the top of the ranking list of harmful alien species in this paper, such as the False indigo (Amorpha fruticosa L.), showed severe and well-documented impacts on the native species composition of invertebrates, plant diversity, and forest regeneration in riparian areas of South-East Europe (Nagy et al. 2018; Kiss et al. 2019), which are challenging to control (Szigetvári 2002; Brigić et al. 2014). Based on the results we suggest to consider including Amorpha fruticosa as invasive species on the EU Union List to facilitate an effective early warning system and rapid eradication measures throughout Europe, where it mainly established in southern EU member states so far. Furthermore, only one invasive plant species causing Major impacts in this study, Heracleum mantegazzianum (rank 22), is ranked among the "more than 100 worst" alien species list for Europe, while two top ranked fungi, Ophiostoma novo-ulmi (rank 29) and Hymenoscyphus fraxineus (rank 18) were identified as species of the greatest concern in Europe (Nentwig et al. 2018). The other identified alien species with high impacts were missed by Nentwig et al. (2018), which indicates that the policy-relevant listing approach is lacking some of the more harmful alien species.

The invasive fungi at the top ranking of this study include globally recognized forest pathogens which parasitize on native trees, such as Ophiostoma novo-ulmi that causes vascular wilt disease of elms known as Dutch elm disease. The disease has resulted in a massive, destructive pandemic in which most of the native elms (Ulmus spp.) have died (Alford and Backhaus 2005; Brunet et al. 2013). Breeding of several resistant clones and reintroduction of resistant native elms mitigated the threat of extinction (Brasier and Webber 2019; Jürisoo et al. 2019; Martín et al. 2019). Another invasive ascomycete fungus, Hymenoscyphus fraxineus, of the high-ranked alien species, causes ash die-back, a lethal disease of ash trees (Fraxinus spp.) in Europe since the early 1990 (Cross et al. 2017; Enderle et al. 2019). The observed impacts on the forests of South-East Europe, including a riparian zone and the generalist nature of the pathogen led to a 'Major' classification of the regionally fast spreading invasive fungus Botryosphaeria dothidea, which causes disease on both native (e.g. Populus spp.) and introduced forest tree species (Jurc et al. 2006; Karadzic et al. 2020; Zlatković et al. 2018). Practical management options for *B. dothidea* and other members of the Botryosphaeriaceae family are limited. Biological control methods against the disease caused by these fungi are being developed, but Botryosphaeriaceae invade xylem vessels thus making the application of pesticides or biological control products difficult or even inefficient (Aćimović et al. 2019; Karličić et al. 2020).

Invasive alien insects on average showed the lowest impacts. This is similar to the only other quantitative cross taxa comparison (based on the Generic Impact Scoring System GISS) which also included non-forest animals and plant species (Kumschick et al. 2015). Most of the insect species in the study area feed on leaves at levels that do not detrimentally affect the performance of the affected trees and only few references

report damage to native trees. For example, the fruit and nut breeding Nearctic insect *Chymomyza amoena* was assigned to the lowest impact category Minor concern (MC), because no negative impact on native host species was observed despite its rapid spread since its arrival to Europe in 1975. However, the impact classification of alien insects may increase in time, if more research on other mechanisms is conducted like the competition with native species, which was recently discussed by Paulin et al. (2020) for North American oak lace bug (*Corythucha arcuata*). The feeding by *C. arcuata* can lead to a shortage of food for specialized oak-associated species and can cause larger negative impacts than previously expected (Paulin et al. 2020). Further, some invasive alien insects with a high negative environmental impact, such as the emerald ash borer (*Agrilus planipennis*), were not included for the EICAT assessment in this study, as the species was not yet found or is expected to currently occur in the Biosphere Reserve.

Alien species from the Northern hemisphere have higher environmental impacts than alien species from the Southern hemisphere. The residence time, measured as the time period that an alien species has been first recorded in Europe, was linked to the origin, especially for plants: alien plants showed an average residence time of 242 years, followed by 62 years for fungi and 60 years of residence time for insects. Alien species from the Northern hemisphere were present in Europe for a longer time period than alien species from the Southern hemisphere. They also occur more frequently, as only 2.5% of the alien species in the study area originate from the Southern hemisphere.

The EICAT classification revealed the impact mechanisms of 85% of the assessed alien species. Two impact mechanisms accounted for 68% of impacts across taxonomic groups: Parasitism for fungi and insects, and Competition for plants. This may partly be due to the different focus of the assessed studies; most references on insects and fungi studied the impact of insects and fungi on the health of their host trees. The assessed impact reports for this study on fungi and insects were mostly published by experts in forest protection, and for plants by experts in invasion biology. This may explain the different focus on the studied impact and impact mechanism of alien species, which impact tree species of economic interests (insects and fungi), and alien species, which impact the species richness (plants). However, the indirect impact mechanisms are more difficult to analyse, therefore impact reports usually focus on studying the direct impacts are chronically underestimated, because the research direction is mainly focussed on the effects of insects on individual trees.

The EICAT classification identified knowledge gaps for 84 alien species, which were assigned to the category 'Data deficiency' (DD). We had to assign species to the category DD for three reasons: Firstly, no references were found on the species; second, references were found, but no impact was described or observed that can be assigned under EICAT; third, references describing impacts were found, but these impacts were not reported from European forest ecosystems. We suggest prioritizing research efforts on alien species with a commonly known impact outside of forests to investigate their potential impact on European forest ecosystems. For example, the invasive alien cicada

Stictocephala bisonia caused plant damage and crop losses in Europe, but the impact on forest ecosystems has not been studied, although the species has been spreading in European forests (Walczak et al. 2018; Hörren et al. 2019). Furthermore, the risk of hybridization and competition of Asian weeping willow (*Salix babylonica* L.) with native species has been reported for forest ecosystems outside Europe, but the impacts were not yet investigated for European forest ecosystems (Amy and Robertson 2001; Richardson and Rejmánek 2011; Thomas and Leyer 2014). For some alien species, valuable references for forests on other continents, which are similar to European temperate forests in ecological conditions, were not included in this study, but could provide interesting results for the prioritization of alien species in forest ecosystems.

Paap et al. (2020) encourages the collaboration of the two disciplines, invasion biology and plant pathology, to increase the success and efficiency for global biosecurity (Hulme 2021). In this study we experienced that interdisciplinary knowledge of the team of assessors is beneficial for cross-taxa EICAT assessments, which increased the understanding of the magnitude of environmental impacts of alien species of different taxonomic groups. The classification of alien species into harmful impact categories is needed for both forest health and invasive species management, as harmful alien species can cause great socio-economic impacts caused by decrease of timber production as well as the increase of management expenses (Hauer et al. 2020). It is therefore highly suggested to do a socio-economic impact assessment with SEICAT (Bacher et al. 2018) in order to include it in further management considerations.

This study has several implications for forests and forestry. Traditionally, forest management in the context of invasive alien species was focused on pests and diseases (Liebhold 2012). Many of them are also invasive alien species with a huge impact on the forest and the potentially harmful ones are listed in the EU regulations as quarantine species (Schrader and Unger 2003). Our study shows that fungi do have a very high environmental impact in forests, but plants are also represented among the highest impacting invasive alien species in the riparian forests of the transboundary Mura-Drava-Danube Biosphere Reserve in Southeast Europe. Therefore, more attention should be paid to invasive plants and the ground layer vegetation.

Conclusions

We see the classification of alien species according to the magnitude of their environmental impact as an important tool for prioritizing the species on which conservationists and forest managers should focus their immediate attention and for policy makers to ensure funding for protecting our forests from invasions. Especially in respect to the high level of biodiversity and heritage value provided in riparian forest ecosystems (Richardson et al. 2007; Ellison et al. 2017) as well as their numerous abiotic and biotic threats, the ranking approach is to be considered complementary to a site-led management approach, where prioritization is driven by urgency of control relative to the extinction of the native species (Downey et al. 2010). We demonstrated that EICAT assessments were useful to prioritize alien species in the local assessment area and to refocus research efforts on recent knowledge gaps. More research on the impacts and impact mechanisms of more recently introduced alien species, especially insects and fungi, is needed to implement effective management measures in the early stage of the invasion. Additionally, analysis of available control methods is another prerequisite for planning conservation activities.

We join the recommendation that EICAT assessments should be performed as transparently as possible, which allows an open discussion of the results (Kumschick et al. 2020). This study is only the second study after Volery et al. (2021) that publishes the original impact data that led to the EICAT classifications. The EICAT assessment can also be repeated after some time, as updated impact evidence can be found or new alien species occur in the region of the assessment area (IUCN 2020a). In conclusion, we recommend applying the EICAT protocol when planning conservation activities, because it decreases the danger of overlooking potential high-risk alien species. Although we are aware that the assessments reported here are a snapshot in time and space and impact magnitudes might change over time, a repeated application of EICAT will be very useful to study spatio-temporal trends in impact magnitudes.

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Appendix I

Table A1. List of the 189 alien species included in the EICAT assessment by the maximum EICAT impact category (EICAT), impact mechanism native range (Origin), and information on the year of introduction in Europe (Years).

| EICAT | Species | Taxonomic | Impact mechanism | Origin | Years |
|----------|--------------------------------|-----------|-------------------------------------|---------------|-------|
| category | | group | | | |
| MR | Biscogniauxia mediterranea | fungi | (5) Parasitism | North America | 1931 |
| MR | Botryosphaeria dothidea | fungi | (5) Parasitism | Europe | |
| MR | Cryphonectria parasitica | fungi | (5) Parasitism | Asia | 1938 |
| MR | Hymenoscyphus fraxineus | fungi | (1) Parasitism | Asia | 1990 |
| MR | Ophiostoma novo-ulmi | fungi | (5) Parasitism | Asia | 1990 |
| MR | Amorpha fruticosa | plants | (1) Competition | North America | 1724 |
| MR | Heracleum persicum | plants | (1) Competition | Asia | 1817 |
| MR | Humulus scandens | plants | (1) Competition | Asia | 1880 |
| MR | Impatiens glandulifera | plants | (1) Competition | Asia | 1839 |
| MR | Reynoutria japonica | plants | (9) Chemical impact on ecosystem | Asia | 1851 |
| MR | Reynoutria sachalinensis | plants | (1) Competition | Asia | 1860 |
| MO | Cucurbitaria piceae | fungi | (5) Parasitism | North America | 1909 |
| МО | Entoleuca mammata | fungi | (5) Parasitism | North America | 1975 |
| MO | Erysiphe alphitoides | fungi | (5) Parasitism | tropical Asia | 1907 |
| MO | Eutypella parasitica | fungi | (5) Parasitism | North America | 1950 |
| МО | Guignardia aesculi | fungi | (1) Competition | North America | 1950 |
| МО | Nothophaeocryptopus gaeumannii | fungi | (5) Parasitism | North America | 1930 |
| МО | Phytophthora alni | fungi | (5) Parasitism | Europe | 1993 |
| МО | Sclerencoelia pruinosa | fungi | (5) Parasitism | North America | 1977 |
| МО | Aphytis mytilaspidis | insects | (5) Parasitism | Asia | 1928 |
| МО | Encarsia berlesei | insects | (11) Structural impact on ecosystem | Asia | 2020 |
| МО | Phyllonorycter issikii | insects | no information | Asia | 1985 |
| MO | Ailanthus altissima | plants | (1) Competition | Asia | 1740 |
| MO | Ambrosia artemisiifolia | plants | (1) Competition | North America | 1863 |
| MO | Artemisia verlotiorum | plants | (1) Competition | Asia | 1873 |
| МО | Asclepias syriaca | plants | (11) Structural impact on ecosystem | North America | 1930 |
| МО | Conyza canadensis | plants | (1) Competition | North America | 1600 |
| MO | Heracleum mantegazzianum | plants | (1) Competition | Asia | 1849 |
| MO | Impatiens parviflora | plants | (1) Competition | Asia | 1831 |
| MO | Iva xanthiifolia | plants | (1) Competition | North America | 1842 |
| МО | Lupinus polyphyllus | plants | (11) Structural impact on ecosystem | North America | 1807 |
| MO | Panicum acuminatum | plants | (11) Structural impact on ecosystem | North America | 1990 |
| MO | Panicum capillare | plants | (11) Structural impact on ecosystem | North America | 1800 |
| MO | Paulownia tomentosa | plants | no information | Asia | 1834 |
| MO | Phytolacca americana | plants | (1) Competition | North America | 1600 |
| MO | Pinus strobus | plants | (11) Structural impact on ecosystem | North America | 1800 |
| MO | Prunus laurocerasus | plants | no information | Asia | 1576 |
| MO | Prunus serotina | plants | no information | North America | 1623 |
| MO | Quercus rubra | plants | (1) Competition | North America | 1700 |
| MO | Reynoutria bohemica | plants | (1) Competition | Europe | 1982 |
| MO | Robinia pseudacacia | plants | (1) Competition | North America | 1601 |
| MO | Solidago canadensis | plants | (1) Competition | North America | 1645 |
| MO | Solidago gigantea | plants | no information | North America | 1700 |
| MO | Spiraea tomentosa | plants | no information | Asia | 1850 |
| MO | Symphyotrichum novi-belgii | plants | (1) Competition | North America | 1865 |
| MO | Ulmus pumila | plants | (3) Hybridisation | Asia | |

| EICAT | Species | Taxonomic | Impact mechanism | Origin | Years |
|----------|--|-----------|---|----------------|-------|
| category | - | group | - | 0 | |
| MN | Apiognomonia veneta | fungi | (5) Parasitism | no information | |
| MN | Blumeriella jaapii | fungi | (5) Parasitism | no information | 1885 |
| MN | Cronartium ribicola | fungi | (5) Parasitism | Asia | 1983 |
| MN | Dothistroma septosporum [as 'septospora'] | fungi | (5) Parasitism | North America | 1960 |
| MN | Drepanopeziza punctiformis | fungi | (5) Parasitism | North America | 1958 |
| MN | Erysiphe arcuata | fungi | (5) Parasitism | North America | 2009 |
| MN | Erysiphe elevata | fungi | (5) Parasitism | North America | 2002 |
| MN | Erysiphe flexuosa | fungi | (5) Parasitism | North America | 2000 |
| MN | Erysiphe platani | fungi | (5) Parasitism | North America | 1960 |
| MN | Glomerella acutata | fungi | (5) Parasitism | Australia | 1990 |
| MN | Guignardia philoprina | fungi | (5) Parasitism | no information | 1970 |
| MN | Lachnellula willkommii | fungi | (5) Parasitism | Asia | 1800 |
| MN | Melampsoridium hiratsukanum | fungi | (5) Parasitism | Asia | |
| MN | Monilinia fructicola | fungi | (1) Competition | Africa | 1970 |
| MN | Mycosphaerella pini | fungi | (5) Parasitism | North America | 1989 |
| MN | Neonectria coccinea | fungi | (5) Parasitism | Europe | |
| MN | Petrakia echinata | fungi | (5) Parasitism | Europe | 1966 |
| MN | Phloeospora robiniae | fungi | (5) Parasitism | North America | 1853 |
| MN | Plectophomella concentrica | fungi | (4) Transmission of disease to native species | no information | 1981 |
| MN | Pseudomicrostroma juglandis | fungi | (5) Parasitism | no information | |
| MN | Rhabdocline pseudotsugae | fungi | (5) Parasitism | North America | 1971 |
| MN | Adelencvrtus aulacaspidis | insects | (5) Parasitism | North America | |
| MN | Aproceros leucopoda | insects | (5) Parasitism | Asia | 2003 |
| MN | Ceroplastes japonicus | insects | (5) Parasitism | Asia | 1983 |
| MN | Corvthucha arcuata | insects | (5) Parasitism | North America | 2000 |
| MN | Drvocosmus kuriphilus | insects | (12) Indirect impacts through interactions | Asia | 2002 |
| | | | with other species | | |
| MN | Halyomorpha halys | insects | (5) Parasitism | Asia | 2007 |
| MN | Hyphantria cunea | insects | (5) Parasitism | North America | 1940 |
| MN | Impatientinum asiaticum | insects | (5) Parasitism | Asia | 1967 |
| MN | Metcalfa pruinosa | insects | (5) Parasitism | North America | 1979 |
| MN | Orientus ishidae | insects | (4) Transmission of disease to native species | Asia | 1998 |
| MN | Parectopa robiniella | insects | (5) Parasitism | North America | 1983 |
| MN | Phyllonorycter robiniella | insects | (5) Parasitism | North America | 1996 |
| MN | Prociphilus fraxinifolii | insects | (5) Parasitism | North America | 2003 |
| MN | Rhagoletis completa | insects | (5) Parasitism | North America | 1990 |
| MN | Xylosandrus germanus | insects | (5) Parasitism | Asia | 1952 |
| MN | Acer negundo | plants | (1) Competition | North America | 1688 |
| MN | Berberis aquifolium | plants | (1) Competition | North America | 1860 |
| MN | Bidens frondosa | plants | no information | North America | 1891 |
| MN | Buddleja davidii | plants | no information | Asia | 1890 |
| MN | Celtis occidentalis | plants | no information | North America | 1785 |
| MN | Hemerocallis fulva | plants | (1) Competition | Asia | 1753 |
| MN | Lonicera iaponica | plants | no information | Asia | 1900 |
| MN | Panicum dichotomiflorum | plants | (1) Competition | North America | |
| MN | Parthenocissus inserta | plants | no information | North America | 1887 |
| MN | Parthenocissus quinquefolia | plants | (10) Physical impact on ecosystem | North America | 1679 |
| MN | Physocarpus opulifolius | plants | (1) Competition | North America | 10/ 2 |
| MN | Phytolacca acinosa | plants | (1) Competition | South America | 2006 |
| MN | Rhus typhina | plants | (1) Competition | North America | 1959 |
| MN | Sporobolus neglectus | plants | no information | North America | .,,,, |
| | -1 | r | | | |

| EICAT | Species | Taxonomic | Impact mechanism | Origin | Years |
|----------|-------------------------------|-----------|--|----------------|-------|
| category | | group | | | |
| MN | Symphyotrichum lanceolatum | plants | (6) Poisoning / Toxicity | North America | 1800 |
| MC | Chymomyza amoena | insects | (5) Parasitism | North America | 1975 |
| MC | Deraeocoris flavilinea | insects | (11) Structural impact on ecosystem | Asia | 1996 |
| MC | Heliothrips haemorrhoidalis | insects | (5) Parasitism | South America | 1833 |
| MC | Myzocallis walshii | insects | (5) Parasitism | North America | 1988 |
| MC | Neodryinus typhlocybae | insects | (11) Structural impact on ecosystem | North America | 1987 |
| MC | Obolodiplosis robiniae | insects | (5) Parasitism | North America | 2003 |
| MC | Oegoconia novimundi | insects | (5) Parasitism | North America | 1980 |
| MC | Abutilon theophrasti | plants | (4) Transmission of disease to native species | Asia | 1800 |
| МС | Artemisia annua | plants | no information | Asia | |
| МС | Catalpa bignonioides | plants | no information | North America | 1726 |
| МС | Gleditsia triacanthos | plants | no information | North America | 1700 |
| MC | Juglans nigra | plants | (9) Chemical impact on ecosystem | North America | 1686 |
| MC | Lonicera maackii | plants | no information | North America | 1896 |
| MC | Oenothera biennis | plants | no information | North America | 1600 |
| MC | Oenothera glazioviana | plants | (3) Hybridisation | North America | 1850 |
| MC | Oxalis dillenii | plants | (12) Indirect impacts through interactions with other species | North America | 1960 |
| MC | Spiraea japonica | plants | (1) Competition | Asia | |
| DD | Ganoderma pfeifferi | fungi | no information | Europe | 1994 |
| DD | Phaeocryptopus nudus | fungi | no information | Asia | |
| DD | Sawadaea tulasnei | fungi | no information | North America | 2012 |
| DD | Volutella buxi | fungi | no information | no information | 1997 |
| DD | Adelges viridula | insects | (5) Parasitism | Asia | |
| DD | Antheraea yamamai | insects | (5) Parasitism | Asia | 1860 |
| DD | Caenoscelis subdeplanata | insects | no information | North America | 2000 |
| DD | Chaetosiphon fragaefolii | insects | no information | South America | 1941 |
| DD | Coccus pseudomagnoliarum | insects | no information | Asia | 2003 |
| DD | Diaspidiotus perniciosus | insects | no information | Asia | 1988 |
| DD | Drosophila suzukii | insects | (5) Parasitism | Asia | 2009 |
| DD | Eriosoma lanigerum | insects | no information | North America | 1787 |
| DD | Glischrochilus quadrisignatus | insects | no information | North America | 1945 |
| DD | Japananus hyalinus | insects | (4) Transmission of disease to native species | Asia | 1961 |
| DD | Myzus ornatus | insects | (5) Parasitism | North America | 1932 |
| DD | Nematus tibialis | insects | (5) Parasitism | North America | 1837 |
| DD | Neoclytus acuminatus | insects | no information | North America | 1908 |
| DD | Neopulvinaria innumerabilis | insects | no information | North America | 1996 |
| DD | Pseudaulacaspis pentagona | insects | no information | Asia | 2005 |
| DD | Pulvinaria hydrangeae | insects | (5) Parasitism | North America | 1965 |
| DD | Saissetia coffeae | insects | no information | Africa | 1977 |
| DD | Stictocephala bisonia | insects | (5) Parasitism | North America | 1912 |
| DD | Trichoferus campestris | insects | (5) Parasitism | Asia | 1967 |
| DD | Xylotrechus stebbingi | insects | no information | Asia | 1952 |
| DD | Abutilon abutiloides | plants | no information | North America | |
| DD | Aesculus hippocastanum | plants | no information | Europe | 1561 |
| DD | Amaranthus powellii | plants | no information | South America | |
| DD | Amaranthus retroflexus | plants | no information | North America | 1700 |
| DD | Armoracia rusticana | plants | no information | Asia | 1514 |
| DD | Broussonetia papyrifera | plants | no information | Asia | |
| DD | Commelina communis | plants | no information | Asia | 1880 |
| DD | Consolida aiacis | plants | no information | Asia | |
| DD | Cotoneaster horizontalis | plants | no information | Asia | 1889 |
| | | 1 | | | |

| EICAT | Species | Taxonomic | Impact mechanism | Origin | Years |
|----------|--------------------------|-----------|------------------|----------------|-------|
| category | | group | | | |
| DD | Cuscuta campestris | plants | no information | North America | 1800 |
| DD | Duchesnea indica | plants | no information | Asia | 1800 |
| DD | Echinocystis lobata | plants | no information | North America | 1904 |
| DD | Elaeagnus angustifolia | plants | no information | Asia | 1633 |
| DD | Eleusine indica | plants | no information | Asia | |
| DD | Epilobium ciliatum | plants | no information | North America | 1891 |
| DD | Erechtites hieraciifolia | plants | no information | South America | 1876 |
| DD | Erigeron annuus | plants | no information | North America | 1700 |
| DD | Erucastrum gallicum | plants | no information | Europe | |
| DD | Euphorbia humifusa | plants | no information | Asia | |
| DD | Euphorbia maculata | plants | no information | North America | 1600 |
| DD | Euphorbia nutans | plants | no information | North America | |
| DD | Fraxinus americana | plants | no information | North America | 1724 |
| DD | Fraxinus pennsylvanica | plants | no information | North America | 1783 |
| DD | Galinsoga parviflora | plants | no information | North America | 1800 |
| DD | Galinsoga quadriradiata | plants | no information | North America | 1892 |
| DD | Glyceria striata | plants | no information | North America | 1849 |
| DD | Helianthus × laetiflorus | plants | no information | North America | |
| DD | Helianthus pauciflorus | plants | no information | North America | 1500 |
| DD | Helianthus tuberosus | plants | no information | North America | 1607 |
| DD | Juncus tenuis | plants | (1) Competition | North America | 1795 |
| DD | Koelreuteria paniculata | plants | (1) Competition | Asia | 1765 |
| DD | Lepidium virginicum | plants | no information | North America | 1713 |
| DD | Lindernia dubia | plants | no information | North America | |
| DD | Lonicera tatarica | plants | no information | Asia | 1770 |
| DD | Lycium barbarum | plants | no information | Asia | 1800 |
| DD | Matricaria discoidea | plants | no information | North America | 1852 |
| DD | Morus alba | plants | no information | Asia | 1600 |
| DD | Oxalis corniculata | plants | no information | North America | 1656 |
| DD | Oxalis stricta | plants | no information | North America | 1800 |
| DD | Panicum miliaceum | plants | no information | Asia | 1700 |
| DD | Platanus × hispanica | plants | no information | no information | 1600 |
| DD | Platycladus orientalis | plants | no information | Asia | 1690 |
| DD | Potentilla indica | plants | no information | Asia | 1800 |
| DD | Reynoutria aubertii | plants | no information | Asia | 1900 |
| DD | Reynoutria baldschuanica | plants | no information | Asia | 1900 |
| DD | Reynoutria multiflora | plants | no information | Asia | |
| DD | Rosa rugosa | plants | no information | Asia | 1796 |
| DD | Rubus armeniacus | plants | no information | Asia | 1835 |
| DD | Rudbeckia laciniata | plants | no information | North America | 1886 |
| DD | Salix babylonica | plants | no information | Asia | 1730 |
| DD | Solanum lvcopersicum | plants | no information | South America | 1544 |
| DD | Solidago gigantea | plants | no information | North America | 1700 |
| DD | Sorghum halepense | plants | no information | Asia | 1914 |
| DD | Symphoricarpus albus | plants | no information | North America | 1800 |
| DD | Tanacetum parthenium | plants | no information | Asia | 1000 |
| DD | Veronica persica | plants | no information | Acia | |
| DD | Vitis vultina | plants | no information | North America | |
| DD | Xanthium alhinum | plante | no information | Acia | |
| | Xanthium orientale | plante | no information | North America | |
| סס | Yanthiam carchanatum | plants | no information | Asia | |
| 50 | xuntinum succharatum | plants | no information | Asia | |

Appendix 2

Table A2. List of concurrence and variance results for each alien species.

| Alien species | Concurrence | Variance |
|--------------------------------|-------------|----------|
| Acer negundo | 66.67 | 0.27 |
| Ailanthus altissima | 60.00 | 0.80 |
| Ambrosia artemisiifolia | 33.33 | 1.00 |
| Amorpha fruticosa | 77.78 | 0.25 |
| Aphytis mytilaspidis | 66.67 | 1.33 |
| Aproceros leucopoda | 83.33 | 0.17 |
| Asclepias syriaca | 100.00 | 0.00 |
| Bidens frondosa | 100.00 | 0.00 |
| Blumeriella jaapii | 100.00 | 0.00 |
| Buddleja davidii | 66.67 | 0.33 |
| Celtis occidentalis | 66.67 | 0.33 |
| Ceroplastes japonicus | 100.00 | 0.00 |
| Chymomyza amoena | 100.00 | 0.00 |
| Conyza canadensis | 100.00 | 0.00 |
| Corythucha arcuata | 100.00 | 0.00 |
| Cronartium ribicola | 100.00 | 0.00 |
| Cryphonectria parasitica | 66.67 | 0.33 |
| Dryocosmus kuriphilus | 100.00 | 0.00 |
| Erysiphe alphitoides | 50.00 | 0.50 |
| Glomerella acutata | 100.00 | 0.00 |
| Halyomorpha halys | 100.00 | 0.00 |
| Humulus scandens | 50.00 | 0.50 |
| Hymenoscyphus fraxineus | 75.00 | 1.00 |
| Impatiens glandulifera | 66.67 | 0.33 |
| Impatiens parviflora | 100.00 | 0.00 |
| Lupinus polyphyllus | 33.33 | 0.80 |
| Metcalfa pruinosa | 75.00 | 0.21 |
| Neodryinus typhlocybae | 100.00 | 0.00 |
| Neonectria coccinea | 100.00 | 0.00 |
| Nothophaeocryptopus gaeumannii | 50.00 | 0.50 |
| Obolodiplosis robiniae | 100.00 | 0.00 |
| Ophiostoma novo-ulmi | 60.00 | 0.21 |
| Panicum acuminatum | 66.67 | 1.33 |
| Panicum capillare | 50.00 | 2.00 |
| Panicum dichotomiflorum | 50.00 | 0.50 |
| Parthenocissus quinquefolia | 75.00 | 0.25 |
| Paulownia tomentosa | 50.00 | 0.50 |
| Phloeospora robiniae | 100.00 | 0.00 |
| Phyllonorycter issikii | 50.00 | 0.50 |
| Physocarpus opulifolius | 66.67 | 0.33 |
| Phytolacca acinosa | 50.00 | 0.50 |
| Phytolacca americana | 50.00 | 0.67 |
| Phytophthora alni | 50.00 | 0.50 |
| Pinus strobus | 100.00 | 0.00 |
| Prociphilus fraxinifolii | 100.00 | 0.00 |
| Prunus laurocerasus | 50.00 | 2.00 |
| Prunus serotina | 100.00 | 0.00 |
| Quercus rubra | 66.67 | 0.33 |
| Reynoutria bohemica | 66.67 | 0.33 |
| Reynoutria sachalinensis | 75.00 | 0.21 |

| Alien species | Concurrence | Variance |
|--------------------------|-------------|----------|
| Reynoutria japonica | 50.00 | 0.92 |
| Rhabdocline pseudotsugae | 100.00 | 0.00 |
| Rhagoletis completa | 100.00 | 0.00 |
| Robinia pseudacacia | 66.67 | 1.33 |
| Sclerencoelia pruinosa | 100.00 | 0.00 |
| Solidago canadensis | 66.67 | 0.24 |
| Solidago gigantea | 45.45 | 0.56 |
| Sporobolus neglectus | 50.00 | 0.50 |
| Ulmus pumila | 50.00 | 2.00 |



First successful hybridization experiment between native European weatherfish (*Misgurnus fossilis*) and non-native Oriental weatherfish (*M. anguillicaudatus*) reveals no evidence for postzygotic barriers

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Abstract

The European weatherfish *Misgurnus fossilis* (Linnaeus, 1758) is a threatened freshwater species in large parts of Europe and might come under pressure from currently establishing exotic weatherfish species. Additional threats might arise if those species hybridize which has been questioned in previous research. Regarding the hybridization of *M. fossilis* \times *M. anguillicaudatus* (Cantor, 1842), we demonstrate that despite the considerable genetic distance between parental species, the estimated long divergence time and different ploidy levels do not represent a postzygotic barrier for hybridization of the European and Oriental weatherfish. The paternal species can be easily differentiated based on external pigment patterns with hybrids showing intermediate patterns. No difference in standard metabolic rate, indicating a lack of hybrid vigour, renders predictions of potential threats to the European weatherfish from hybridization with the Oriental weatherfish difficult. Therefore, the genetic and physiological basis of invasiveness via hybridization remains elusive in *Misgurnus* species and requires further research. The existence of prezygotic reproductive isolation mechanisms and the fertility of F1 hybrids remains to be tested to predict the potential threats of globally invasive Oriental weatherfish species.

Keywords

Freshwater fish, genome size, pigment patterns, postzygotic barrier, standard metabolic rate

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Introduction

Freshwater fish biodiversity is declining at an alarming rate (Reid et al. 2019; Tickner et al. 2020). Among other factors, habitat loss and invasive alien species are major drivers of species extinction (Clavero and Garcia-Berthou 2005; Cucherousset and Olden 2011; Closs et al. 2016). Wetlands are disappearing worldwide due to low agricultural value, facilitating conversion to arable land or other land "developments". Fishes specifically adapted to such habitats and serving as indicator species for the ecological status of wetlands, e.g. mudminnows (*Umbra krameri*, Walbaum 1792) (Wanzenböck 2004), are particularly affected (Tickner et al. 2020). Especially if habitat loss is combined with threats from closely related invasive species, effects might be non-additive and massive (Clavero and Garcia-Berthou 2005; Didham et al. 2007). Such a scenario is currently developing for the European weatherfish (*Misgurnus fossilis*, Linnaeus 1758) and the closely related, invasive Oriental weatherfish (*Misgurnus anguillicaudatus* (Cantor, 1842)).

The European weatherfish is a native species distributed across Europe and western Asia and is of significant conservation concern (Bohlen et al. 2007; Freyhof 2013). It is included in the list of freshwater fish species requiring international protection (European Union Habitats Directive and Bern Convention, see https://eunis.eea.europa.eu/ species/551#legal_status) and on a number of national red lists (Wolfram and Mikschi 2007). Declining populations are primarily based on habitat loss (Wolfram and Mikschi 2007; Brys et al. 2020b) because the European weatherfish is specifically adapted to floodplain backwaters and wetlands of large rivers (Ràb et al. 2007). However, the invasion by the closely related Oriental weatherfish might contribute to the disappearance of European weatherfish in the future (Riffel et al. 1994). The Oriental weatherfish was imported as early as 1870 to Hawaii by Asian immigrants using it as food fish (Nico et al. 2019), and to the rest of the USA in the late 19th century by the ornamental pet fish industry as an aquarium and garden pond fish (Maceda-Veiga et al. 2013; Nico et al. 2019). Established populations in the wild have been found in many parts of the world (Milton et al. 2018) including Australia, Europe, South America (Abilhoa et al. 2013) and the USA (Frable 2008; Nico et al. 2019). In Europe, feral exotic weatherfish were initially found in Germany by Riffel et al. (1994) reporting, besides their genetic study on fish from a pond, a wild individual of *M. mizolepis* (Günther) being caught in the Nahe river (Rhineland-Palatinate) in 1993. Subsequently, Oriental weatherfish were recorded in Italy (Razzetti et al. 2001), Germany (Freyhof and Korte 2005), Spain (Franch et al. 2008), the Netherlands (van Kessel et al. 2013), from where it presently expands into Belgium, (Verreycken pers. comm), to Southern Germany (Belle et al. 2017), and recently to Austria (Zangl et al. 2020). However, there is some discrepancy regarding the taxonomy of different populations of exotic weatherfish found in Europe belonging to M. anguillicaudatus, M. bipartitus (Sauvage & Dabry de Thiersant, 1874), Paramisgurnus dabryanus (Dabry de Thiersant, 1872) or M. mizolepis (Günther, 1888) (Milton et al. 2018; Zangl et al. 2020; Belle et al. 2021).

A high probability of direct contact of the European and Oriental weatherfish in the same waterbody can be foreseen in the near future because of similar habitat preferences

(Meyer and Hinrichs 2000; Frable 2008; Brys et al. 2020a), however, predictions of potential consequences for the native species are difficult. Apart from competition based on broadly overlapping ecological niches, threats are predictable based on close taxonomic relationship, facilitating hybridization (Riffel et al. 1994; Rhymer and Simberloff 1996; Rhymer 2008). In the case of successful hybridization, one could anticipate high risks based on competitive advantages of hybrids (Huxel 1999; Rosenfield et al. 2004; Schierenbeck and Elstrand 2009; Coulter et al. 2020), especially if the hybrids show heterosis effects. On the other hand, risks from hybrids might be low if hybrids are not viable or are infertile representing a post-zygotic isolation mechanism (Janko et al. 2017).

Therefore, we tested in a primarily qualitative study for 1) the presence/absence of postzygotic reproductive isolation measured as embryo/larval viability by conducting a reciprocal hybridization experiment, and 2) the presence/absence of heterosis effects manifested in a physiological trait such as standard metabolic rate in relation to genome size and/or ploidy levels.

Material and methods

Fish propagation

Eight adult individuals of the European native species (European weatherfish, *M. fos-silis*, see Suppl. material 1: Fig. S1, Suppl. material 2: Fig. S2) were obtained from a wild population in the floodplain of the River March (=Morava, close to Baumgarten) forming the border between Austria and Slovakia. Electrofishing was used on March 29, 2018, to catch adult fish intended primarily for artificial reproduction and restocking of juveniles within a conservation project. They were transferred to Mondsee, Austria, and held in a large aquarium (350 Liter) for 8 weeks. Eggs of one large (23 cm total length) female (out of 4 females) and sperm from 3 adult males (out of 4 males) were used for the hybridization experiments.

Five adult fish of the non-native species *M. anguillicaudatus*, (see Suppl. material 3: Fig. S3, Suppl. material 4: Fig. S4) were obtained from a garden pond population in summer 2017. 10 morphometric parameters following Yi et al. (2017), identified them as *M. anguillicaudatus* and discriminated them from *M. bipartitus*, another exotic weatherfish species found in Europe (Zangl et al. 2020). The most discriminating ratio values (i.e. caudal peduncle length/body length, caudal peduncle height/caudal peduncle length) were found to be 0.163 ± 0.015 (mean \pm SD) and 0.619 ± 0.085 , respectively, and were always closer to the values given for *M. anguillicaudatus* than to *M. bipartitus* (table 1 in Yi et al. 2017). A misidentification with *P. dabryanus* can be excluded due to our genome size measurements which show a C-value of approx. 1.65 pg/nucleus. According to "Animal genome database" (T. Ryan Gregory https://www.genomesize.com/results.php?page=1) diploid *M. anguillicatudatus* are in the range of 1.37-1.86pg/nucleus. Furthermore, misi-

dentification with *M. bipartitus* can be excluded based on genetic identity analysis of the mitochondrial 12S marker according to Miya et al. (2015). Our individuals have been sequenced using MiFish primers and showed percent identity values of 96.55–99.43 to *M. anguillicaudatus* sequences published in NCBI, whereas they showed values of only 93.6% to *M. bipartitus*. Percent identity values to *P. dabry-anus* showed values of 86.71–87.86.

Non-native fishes were held in a large (400 Liter), unheated aquarium over the autumn 2017 and winter 2017/2018. Eggs of two females (18 and 22 cm total length respectively) and sperm of 3 adult males were used for the experiments. The aquarium was in an unheated room and was exposed to natural temperature rise in spring 2018, and natural daylight from a large window in close proximity. Three weeks before hormone treatment was initiated (May 25), both aquaria inhabited by native and alien weatherfish respectively, were heated using aquarium heaters from 16 °C to 21 °C at a rate of 1 °C every other day.

Hormone treatment was performed following Kouril et al. (1996) and Schreiber et al. (2017) using Ovopel (http://ovopel.hu/en/). Brood fish were injected by Ovopel solutions into the dorsal muscle according to manufacturer's instructions (1 pellet per kg body weight) and gametes were stripped 48 hours later (May 27). Eggs were artificially inseminated in all four reciprocal mating combinations, i.e. eggs of one European weatherfish female (approx. 4,000) were divided in half and one portion was inseminated with mixed sperm of three European weatherfish males, the second portion with mixed sperm of three Oriental weatherfish males. Similarly, mixed eggs of two Oriental weatherfish females (approx. 4,000) were divided in half and one portion fertilized with mixed sperm of three Oriental weatherfish males, the second portion with mixed sperm of three European weatherfish males. Fertilized eggs were incubated in jars which were placed in a 60 L aquarium (21 °C) and aerated. Larvae hatched the next day (May 28). Unfortunately, we obtained only very few hybrid larvae of the mating between female *M. anguillicaudatus* and male *M.* fossilis due to accidental clogging of the incubation jar causing detrimental oxygen shortage. The groups of larvae were kept in separate 30 L aquaria each, containing lake water at a temperature 24 °C \pm 2 °C, supplied with artificial light (day: night 12 h:12 h). The few hybrid larvae produced from Oriental weatherfish females and European weatherfish males (estimated 5-10 individuals) disappeared during the following three days - the last larva observed was killed by a large copepod introduced accidentally into the aquarium, thus excluding this group from further analysis. Eggs (estimated to be 1,000-2,000 per group) and larvae developed normally in the other three groups, similar to previous artificial reproduction campaigns of M. fossilis, photographically documented in Schauer et al. (2013). Animals were fed ad libitum with dry feed, living Artemia nauplii (daily), and chopped worms (Tubifex) (once a week). In those groups, we obtained several hundred (>500) hatched larvae resulting in 200-500 juveniles each in autumn 2018. Exact quantification of survival rates in eggs and larvae/juveniles was not performed, and sex determination is not possible before maturity is reached (2 years).

Standard metabolic rate (SMR)

The Standard Metabolic Rate (SMR) is defined as the minimal amount of oxygen needed to maintain the aerobic metabolism. There are eight different definitions on how to estimate the SMR (Chabot et al. 2016). A time and cost-effective way, and one of the most common estimates, is to take the average of the 10% lowest oxygen consumption values (one value for each 15 min. measuring cycle) during the measurement period (36 hours) after removing the five lowest ones as outliers (low 10%). This analysis was used here as follows: Three acrylic respirometer chambers with a volume of 133 ml each were submerged in a basin filled with 25 L of aerated and treated (sand filter, UV disinfection) lake water (100% oxygen saturation; temperature 20 °C). Water temperature was controlled by a heating (Lauda "Alpha", Lauda, Regensburg, Germany) and a cooling unit (Lauda chiller). A Cyclobios respirometer controller was used to trigger the measuring intervals and flushing periods. Each measurement and flush interval was set to 15 min. Each cycle included three measurement intervals (one for each chamber) followed by one flush interval for all chambers simultaneously. One pump was used to flush the chambers, a second one pumped water from one chamber to an optical Oxygen sensor (YSI-Pro ODo, ecoTech, Bonn, Germany) during the measuring period and back to the chamber in a closed loop. The flow rate was 400 ml/min. In all experiments, fish were starved for 24 h before introducing them into the chamber. One chamber was left empty to determine bacterial oxygen consumption as baseline control. Before measurements, fish were acclimatized in the chamber for 12 hours. Each experiment lasted for 48 hours (including 12 hours of acclimation time) under the natural light regime. All individuals were juveniles and their wet weight ranged between 0.122 g and 1.98 g (mean: 0.65 g + 1 - 0.416 g SD). If fish weight was less than one gram, measurements were conducted in groups of up to six individuals. For weights above one gram, the fish were measured individually to keep biomass in the chambers relatively constant following standard procedures (Chabot et al. 2016). The number of replicates (N in Figs 6 and 7) refers to single chambers and not individuals in the experiments. Statistical comparisons among groups (M. fossilis, M. anguillicaudatus, and hybrids) were done with SigmaPlot (Systat Software, Inc., version 14.0) applying Analysis of Covariance (ANCOVA) using average individual fresh weight as covariable.

Ploidy

Relative genome size was determined in 10 individuals of each group, following the protocol of Lamatsch et al. (2000). Isolated nuclei of fin clips (taken for identification purposes) from juveniles (6 months old) were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich, Vienna, Austria) and measured for fluorescence intensity with a violet laser (405 nm) in a flow cytometer with acoustic focusing technology (Attune NxT, ThermoFisher Scientific, Vienna, Austria) using chicken red blood cells as internal standard (2.5 pg per nucleus; Vinogradov 1998). Ploidy levels were assessed in comparison to relative genome sizes given in Drozd et al. (2010).



Figure 1. Pictures of weatherfishes taken one month after hatching (30.6.18) when larvae transformed into juveniles. Top panel: *M. fossilis* with larval fin fold still present. Center panel: Hybrid weatherfish with rests of larval fin fold present. Lower panel: *M. anguillicaudatus* – only small remnants of larval fin fold visible. Photographs: Sylvia Wanzenböck.

Results

Larvae developed normally without any apparent morphological aberrations in all three groups (*M. fossilis, M. anguillicaudatus*, and hybrids produced from *M. fossilis* females and *M. anguillicaudatus* males). Different pigmentation patterns for these two parental species became apparent during the first weeks of development, with hybrids showing intermediate pigmentation (Figs 1–4). Development to juveniles, judged from the disappearance of the larval fin-fold, occurred approximately 3 weeks later in *M. fossilis* compared to *M. anguillicaudatus* (when 50% individuals in the groups passed this stage) and was intermediate in hybrids.

The lateral side of *M. fossils* can be separated into 4 distinguishable color bands. The overall dorsal part is dark brown. The first band is light yellowish colored and clearly separates from the dark brown band along the lateral line. Ventral to the brown lateral line band another light yellowish color band follows. A thin but distinct dark brown color band separates the ventral side (brown to yellow or sometimes a slightly red color) from the second light yellowish color band. In all dark color bands, several small dark spots might be present. The dorsal dark brown color line on the lateral side can be dispersed into a loose line of brown dots and may end just below the dorsal fin. The most ventral brown color band extends from the basis of the pectoral fins to



Figure 2. Pictures of weatherfishes taken two months after hatching (21.7.18) when development to juveniles was nearly completed. Top panel: *M. fossilis* with larval fin fold still visible. Center panel: Hybrid weatherfish without larval fin fold. Lower panel: *M. anguillicaudatus* – juvenile and no larval fin fold visible. Photographs: Sylvia Wanzenböck.

the pelvic fins, and in some individuals to the basis of the anal fin. This band can be dispersed into a loose line or an interrupted row of brown dots.

The hybrids of *M. fossils* and *M. anguillicaudatus* show a brown-yellowish speckled overall pattern on the dorsal (and part of the lateral) side. The edge of this pattern often consists of a more or less continuous alignment of dark brown dots. It stretches from the eye to the base of the caudal peduncle. Often there is a loose and interrupted row of dots between the base of the dorsal fin and the caudal peduncle. The first color band is light yellowish and filled with several small dark brown dots. It stretches from the gill openings to the base of the caudal peduncle and contains various numbers of small brown dots, which increase in number with age. This color band is not as distinct as in *M. fossilis.* A dark brown color band along the lateral line from the gill openings to the base of the caudal peduncle forms the second color band. This one is also not as clearly distinct as in *M. fossilis*. The third light yellowish band, just next to the second one, is often filled with fine brown dots, and stretches from the base of the pectoralis to the base of the caudal peduncle. This more or less strongly dotted yellowish line is sometimes not clearly distinguishable from the fourth color band which is often dispersed into an interrupted and incomplete alignment of big brown dots. It extends from the basis of the pectoral fin to the ventral fin and in some individuals until the basis of the anal fin.



Figure 3. Pictures of weatherfishes taken three months after hatching (26.8.18) when development to juveniles was completed. Top panel: *M. fossilis*. Center panel: Hybrid weatherfish. Lower panel: *M. anguillicaudatus*. Photographs: Lukas Fuxjäger.

The basic coloration of *M. anguillicaudatus* varies from gray to sand-colored. On the dorsal and lateral side of the body more or less clearly visible dark dots are recognizable in an irregular pattern. Individuals with a high number of dark dots on the lateral side show a thin light band along the lateral line from the gill openings to the base of the caudal peduncle. Just before the dark bow on the very end of the caudal peduncle (sometimes divided, forming a dorsal and ventral black dot), a light half-moon shaped line is located. But there are also individuals without any dots. Pigmentation patterns were variable between individuals (Fig. 5).

Standard metabolic rate (SMR) was found to depend negatively on fresh weight even when oxygen consumption values were calculated per unit weight (gram fresh weight – Fig. 6). Nevertheless, regressions of groups (equal slopes, Fig. 6) and average values (Fig. 7) were statistically not significantly different between parental species and hybrids (ANCOVA, fresh weight as covariable, p = 0.667)

Measurements of ploidy levels revealed relative genome sizes of 5.20 (± 0.123) pg/ nucleus, 4.77 (± 0.07), and 3.33 (± 0.03) on average for *M. fossilis*, M. hybrids and *M. anguillicaudatus*, respectively (Fig. 8). Differences between groups were found to be statistically significant (ANOVA, p < 0.001), and pairwise comparisons (Holm-Sidak method) revealed significant differences among all group pairs (p < 0.001). Since these measure-


Figure 4. Pictures of weatherfishes taken four months after hatching (1.10.18) showing advanced juvenile stages. Top panel: *M. fossilis*. Center panel: Hybrid weatherfish. Lower panel: *M. anguillicaudatus*. Photographs: Sylvia Wanzenböck.

ments are consistent with genome size estimates and chromosome counts of Drozd et al. (2010, see discussion), we categorized *M. anguillicaudatus* as diploid (2n), and *M. fossilis* as tetraploid (4n). The hybrids, however, showed an average genome size of 4.77 (\pm 0.07) pg/ nucleus which is 0.5 pg higher than the expected average between the two parental species.

Discussion

Based on a genetic study using enzyme electrophoresis, Riffel et al. (1994) assumed that hybridization between European and Asian weatherfish might be hampered due to their genetic distance (Nei) of 0.9651, and a difference in ploidy level. The relatively high estimated divergence time between the two species (15–25.5 MYA; www.timetree.org; Tang et al. 2008) supports this hypothesis. We found, however, no postzygotic barrier in embryo survival or larval viability for interspecific F1 hybrids. Whether hybridization is qualitatively possible in both directions (*M. fossilis* females and *M. anguillicaudatus* males and vice versa) remains to be studied. However, based on our observations and hybridization studies in the weatherfish group (e.g. *M. anguillicaudatus* × *P. dabryanus*,



Figure 5. Pictures of weatherfishes taken four months after hatching (1.10.18) showing advanced juvenile stages and individual variation in pigmentation patterns. Left column: *M. fossilis*. Center column: Hybrid weatherfish. Right column: *M. anguillicaudatus*. Photographs: Sylvia Wanzenböck.

Huang et al. 2017) it seems very likely, given that the low survival of eggs and larvae of the hybrids produced from *M. anguillicaudatus* females and *M. fossilis* males could be linked to accidental events. However, we cannot yet determine if hybridization probability and hybrid viability are equal in both directions, or if embryonic and larval mortality is quantitatively asymmetric. Larval development in hybrid individuals was basically similar to pure species and successful. However, there was an indication of elevated malformations in hybrids (personal observation). Determining if hybrids attain sexual maturity and are fertile would be extremely important to assess the potential for hybrid swarms threatening wild populations of the native species (Rhymer 2008) and for our general understanding of the relationship between asexuality, hybridization, and speciation in fishes (Lamatsch and Stöck 2009; Janko et al. 2017).

Differentiation between *M. fossilis* and various exotic weatherfish (in our case *M. anguillicaudatus*) was found to be easily possible based on pigmentation patterns (see also Kottelat and Freyhof 2007). In concordance to other authors (e.g., Freyhof and Korte 2005; Kottelat and Freyhof 2007), we found that species identification is clearcut based on longitudinal, characteristic and alternating dark and light pigment bands in *M. fossilis* which are absent in *M. anguillicaudatus* and other non-native species. We agree that the black dot in the dorsal part of the caudal base is an unsuitable characteristic-



Figure 6. Standard metabolic rate (measured as oxygen uptake per gram fresh weight per hour of juvenile *M. fossilis* (N = 8), juvenile hybrids (N = 7) and juvenile *M. anguillicaudatus* (N = 9) versus fresh weight (average of lowest 10% measurements over time \pm SD). Note that N in this figure refers to number of chambers.

tic for differentiation of *M. fossilis* vs. *M. anguillicaudatus*, as a black dot is also found in *M. fossilis*, at least in our juveniles (see Fig. 5). However, we want to stress that genetic methods, although reliable in *Misgurnus* (e.g., Miya et al. 2015; Yi et al. 2017; Belle et al. 2021) are not the only tool to correctly differentiate the native European weatherfish from introduced Oriental weatherfish, and identification should be possible by trained experts using morphological characters i.e., pigmentation patterns. Anyhow, the discrimination of each of the parental species versus their hybrids, solely on pigmentation patterns may seem difficult although it looked quite obvious to us (Fig. 5).

Our estimates of SMR, measured as oxygen consumption (VO2), were comparable to previous estimates in a closely related loach species, i.e. the spined loach, *Cobitis* sp. (Maciak et al. 2011). Our obtained average values of approximately 0.1 mg O_2 g⁻¹ h⁻¹ are higher compared to their values of approx. 0.05 mg g⁻¹ h⁻¹. However, lowest values are similar (Fig. 6). Given the fact that VO2 values are generally highly variable and are mostly presented as logarithmically transformed values, we consider those values to correspond nicely. Our fish were too large and methods were too different for making meaningful comparisons with measurements on early larval European weatherfish by Schreiber et al. (2017), or those for Oriental weatherfish by White et al. (2017).



Figure 7. Average Standard metabolic rate (\pm SD) measured as oxygen uptake per gram fresh weight per hour for juvenile *M. fossilis* (N = 8), juvenile hybrids (N = 7) and juvenile *M. anguillicaudatus* (N = 9). Note that N in this figure refers to number of chambers.

We observed a negative trend in VO2 per gram wet mass with increasing body mass, which is a well – known pattern in the early ontogeny of larval and juvenile fish (Chabot et al. 2016) and reflects lower weight-specific metabolic rates with increasing body mass as the scaling exponent between oxygen consumption and mass is typically lower than one (Clarke and Johnston 1999; Isaac and Carbone 2010). The decreasing trends were similar for parental species and hybrids.

The relationship between metabolic rates and heterosis (or hybrid vigor, specifically in growth, i.e. "metabolic heterosis" *sensu* Ginn (2017)), does not show clear-cut trends: While decreased (i.e. more efficient) metabolic rates in hybrids are widely found in germinating plants (Sinha and Khanna 1975; Goff 2011), especially in crops, and could be expected based on molecular and cell physiological principles (Chen 2010, 2013; Goff 2011; Ginn 2017; Govindaraju 2019), the findings in animals are controversial. In marine bivalves Hedgecock et al. (1996) observed more efficient protein metabolism coupled with lower oxygen consumption in three out of four replicate experiments, whereas in geckos lower SMR was observed in hybrids in only one out of three regions (Kearney and Shine 2004). In fish, hybridization is widespread (Chevassus 1983; Scribner et al. 2000) and used mainly for aquaculture purposes (Wang et al. 2019). Lower metabolic rates and thus more efficient protein turnover rates were found in more het-



Figure 8. Relative genome sizes (DAPI stained) of individual fishes (N = 10 for each group) in pg/nucleus (average \pm SD) relative to chicken red blood cells (2.5 pg/nucleus, Vinogradov 1998).

erotic individuals within a single strain of rainbow trout, i.e. intraspecific (Danzmann et al. 1988), and in hybrid bass compared to pure striped bass, i.e. interspecific (Tuncer et al. 1990). However, there are also many negative reports of hybrid strains showing no useful heterotic traits for breeding, e.g. chinook salmon (Bryden et al. 2004). In conclusion, the effect of heterosis and its relationship to physiological traits such as SMR in animals and specifically in fish remains elusive and is constrained by a low number of studies measuring those traits in intra- and interspecific hybrids. Therefore, we need more studies to test for genotype-phenotype relationships (Fiévet et al. 2018) involving not only the ultimate outcome such as growth and biological success (invasiveness) but also the underlying characters such as metabolic rates and physiological performance. Anyhow, there are several examples for interspecific hybrids showing heterotic, superior performance compared to parental species (e.g. in *Misgurnus*, Luo et al. 2021). Furthermore, there are cases where hybrids are threatening e.g. Pecos pupfish (Rosenfield et al. 2004) or showing superior invasiveness e.g., hybrid Chinese carps (Coulter et al. 2020), shiners (Blum et al. 2010), or bleak (Almodóvar et al. 2012).

Since representatives of the genus *Misgurnus* were found to show various ploidy levels (Ràb et al. 2007 and citations therein), correlating negatively with SMR (Maciak et al. 2011), ploidy needs to be considered to correctly interpret our results. *M. fossilis* from a population in the Czech Republic (158 km away from the population studied



Figure 9. Example of flow cytometer measurements. The first peak from the left is the internal standard (chicken red blood cells; similar in all measurements). The following peaks represent *M. anguillicaudatus* (green), hybrids (red), *M. fossilis* (purple).

here, but from the Elbe drainage) were found to be dominated by tetraploid individuals (66%, Drozd et al. 2010). However, triploid and aneuploid individuals were also detected (17% each). Ploidy status was confirmed by karyotyping, and the c-values for diploid cells (gametes) were found to be 2.02 pg/nucleus, slightly lower than the estimate of 2.6 pg/nucleus (Timofeeva and Kafiani (1964), cited in Animal Genome Size data base from Gregory (2020)). However, the database value corresponds to our relative genome sizes of 5.2 pg/nucleus for somatic cells. Because of this correspondence, we categorized our *M. fossilis* individuals as tetraploids. C-values of diploid *M. anguillicaudatus* (2n = 50 chromosomes, e.g. Zhang and Arai 2003) range from 1.37 pg (Park and Chung 1985) or 1.4 pg (Hinegardner and Rosen 1972) to 1.86 pg (Suzuki 1992). The C-values of Drozd et al. (2010) for 1n cells of diploid *M. anguillicaudatus* (1.65) correspond well with our average value of 3.3 pg/nucleus for 2n cells. This confirms our classification of *M. anguillicaudatus* being diploid. Hybrids showed an intermediate relative genome size and are therefore presumably triploid (3n). However, their genome size was slightly higher than the mean value of the two parental species. This might be due to the already observed aneuploidy and/or microchromosomes which can only be resolved by karyotyping.

Higher metabolic rates were found to correlate negatively with ploidy level in another loach species, i.e. spined loach, by Maciak et al. (2011). Because of this, we expected lower metabolic rates in the tetraploid European weatherfish compared to the diploid Oriental weatherfish. However, this was not apparent in our dataset. On the contrary, there was a slight, though insignificant, tendency of a direct relationship between ploidy level and metabolic rate, i.e. the diploid Oriental species had a slightly lower SMR compared to the tetraploid European species with triploid hybrids showing intermediate SMR values (Fig. 7 and Fig. 8). The definitive lack of lower metabolic rates in the European weatherfish might be interpreted as a hint towards the secondary rediploidization in *M. fossilis* (Spóz et al. 2017).

Conclusion

We showed that there is no postzygotic barrier for hybridization between the European and Oriental weatherfish despite the assumption given by Riffel et al. (1994) that genetic introgression might be hampered by the large genetic distance (D = 0.9651), and ploidy level differences of the parental species. The existence of prezygotic reproductive isolation mechanisms and fertility of F1 hybrids remains to be tested. A lack of observable heterosis effects (hybrid vigour) in standard metabolic rate (SMR) renders predictions of potential augmented threats to the European weatherfish from hybridization with an Oriental weatherfish difficult. Therefore, the genetic and physiological basis of invasiveness via hybridization remains elusive within the genus *Misgurnus* species and requires further research. However, the European weatherfish is most probably threatened by the globally invasive Oriental weatherfish through competition, even without hybridization. A fact that should be considered in managing and conserving natural freshwater fish biodiversity in Europe.

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Supplementary material I

Figure S1

Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch

Data type: Jpg file

Explanation note: Female Misgurnus fossilis used in the crossing experiment.

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Link: https://doi.org/10.3897/neobiota.69.67708.suppl1

Supplementary material 2

Figure S2

Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch

Data type: Jpg file

Explanation note: Males of Misgurnus fossilis used in the crossing experiment.

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Link: https://doi.org/10.3897/neobiota.69.67708.suppl2

Supplementary material 3

Figure S3

Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch

Data type: Jpg file

Explanation note: Female Misgurnus anguillicaudatus used in the crossing experiment. Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/neobiota.69.67708.suppl3

Supplementary material 4

Figure S4

Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch

Data type: Jpg file

Explanation note: Male Misgurnus anguillicaudatus used in the crossing experiment.

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RESEARCH ARTICLE



What doesn't kill you doesn't make you stronger: Parasites modify interference competition between two invasive amphipods

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Abstract

We used a freshwater amphipod-microsporidian model (Ponto-Caspian hosts: *Dikerogammarus villosus* and *D. haemobaphes*, parasite: *Cucumispora dikerogammari*) to check whether parasites affect biological invasions by modulating behaviour and intra- and interspecific interactions between the invaders. We tested competition for shelter in conspecific and heterospecific male pairs (one or both individuals infected or non-infected). In general, amphipods of both species increased their shelter occupancy time when accompanied by infected rather than non-infected conspecifics and heterospecifics. Infected amphipods faced lower aggression from non-infected conspecifics. Moreover, *D. villosus* was more aggressive than *D. haemobaphes* and more aggressive towards conspecifics vs. heterospecifics. In summary, infection reduced the intra- and interspecific competitivity of amphipods, which became less capable of defending their shelters, despite their unchanged need for shelter occupancy. *Dikerogammarus haemobaphes*, commonly considered as a weaker competitor, displaced by *D. villosus* from co-occupied locations, was able to compete efficiently for the shelter with *D. villosus* when microsporidian infections appeared on the scene. This suggests that parasites may be important mediators of biological invasions, facilitating the existence of large intra- and interspecific assemblages of invasive alien amphipods.

Keywords

aggression, Amphipoda, biological invasions, coexistence, *Dikerogammarus*, interference competition, Microsporidia, parasitic infection

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Introduction

Animal behaviour is known to be modulated by parasites, simply by their pathogenicity and inducing defence responses in their hosts (Satinoff 2011; Żbikowska and Cichy 2012), but also by increasing parasites' fitness through host manipulation (Bakker et al. 1997; Lagrue et al. 2007; Flegr 2015). Parasite-induced changes are usually multidimensional, altering multiple phenotypic traits by a single parasite (Cezilly and Perrot-Minnot 2010; Cezilly et al. 2013). This includes such aspects as morphology (Bakker et al. 1997), habitat selection (Żbikowska and Cichy 2012; Rachalewski et al. 2018), mobility (Dezfuli et al. 2003), boldness (Flegr 2015), aggression (Thomas et al. 2005; Mikheev et al. 2010), foraging (Fielding et al. 2003; Bącela-Spychalska et al. 2014) and reproduction (Hall et al. 2007). These changes may further propagate to the impact on ecosystem functioning (Friesen et al. 2017; Anaya-Rojas et al. 2019) through intra- and interspecific interactions of infected individuals with other organisms through consumption (Fielding et al. 2003), predation (Bakker et al. 1997; Flegr 2015; Friesen et al. 2019) or competition (Anderson and May 1986; Mikheev et al. 2010; Reisinger et al. 2015; Friesen et al. 2018).

Through these mechanisms, parasites may indirectly affect the process of biological invasions (Hatcher et al. 2015), which are considered as one of the most important threats to global biodiversity (Lambertini et al. 2011). Knowledge of ecology of invasive alien species, including their interactions with parasites, is crucial to understand the functioning of ecosystems in the present world (Dunn 2009; Dunn et al. 2012; Roy et al. 2016). Alien species in their novel areas may "escape" from their sympatric parasites and, in accordance with the enemy release hypothesis, get advantage over local biota, suffering standard levels of parasite infestation (Colautti et al. 2004; Dunn 2009; Heger and Jeschke 2014). On the other hand, parasites, both originating from the native range and locally acquired, may limit the spread of the alien hosts (Bojko et al. 2018; Chalkowski et al. 2018) by reducing their competitive ability. A more subtle influence of parasites may consist of modifications of the impact imposed by alien species on local communities (Dunn 2009) by changing their behaviour, e.g. food acquisition or preferences (Bącela-Spychalska et al. 2014; Iltis et al. 2017).

Alien species interfere not only with the local biota, but also with one another as competitors (Dick and Platvoet 2000), prey/predators (Borza et al. 2009), habitat engineers providing shelters (e.g. mussels, Kobak et al. 2009) and sources of interspecific semiochemicals (Rachalewski et al. 2019). The outcome of these interactions can be coexistence, displacement or facilitation. Facilitation, if prevailing at the community level, can contribute to the phenomenon of invasional meltdown (Simberloff and von Holle 1999). Parasites may mediate these interactions and affect their outcomes.

A perfect model to study multi-species interactions among invasive alien species and their parasites is the freshwater assemblage of Ponto-Caspian amphipod crustaceans and their intracellular microsporidian parasites (Bojko and Ovcharenko 2019). These amphipods interfere with invaded European environments as predators of invertebrate fauna (Krisp and Maier 2005), shredders (Truhlar et al. 2013), food sources

for fish (Grabowska and Grabowski 2005; Borza et al. 2009) and competitors of their local relatives (Dick et al. 2002). They occupy similar ecological niches (Dedju 1980), which makes them natural competitors among themselves (e.g. van Riel et al. 2007; Platvoet et al. 2009b; Jermacz et al. 2015), but they can also prey on one another (intra-guild predation) (Dick and Platvoet 2000; Kinzler et al. 2009) and communicate interspecifically, e.g. perceiving heterospecific alarm cues (Rachalewski et al. 2019). In their native region, these amphipods are infected with several species of Microsporidia (Wattier et al. 2007; Ovcharenko et al. 2009, 2010; Bacela-Spychalska et al. 2018), which hitch-hiked with their hosts to novel regions in Europe (Wattier et al. 2007; Ovcharenko et al. 2010; Bącela-Spychalska et al. 2012, 2018; Grabner et al. 2015). These parasites may reach high prevalence, up to 72% (Ovcharenko et al. 2010; Bojko et al. 2015; Iltis et al. 2017) and high ecological importance. Some of them can affect their host's behaviour, such as activity and predation (Bacela-Spychalska et al. 2014; Farahani et al. 2021). The strength of parasite impact depends on the transmission mode: horizontal, vertical or both (Dunn et al. 2001; Bacela-Spychalska et al. 2014; Bojko et al. 2018; Rachalewski et al. 2018). In general, the horizontal transmission is often linked to high virulence (Fielding et al. 2005), while vertical transmission is associated with low or no virulence, or even with the increased host fitness (e.g. Slothouber Galbreath et al. 2004). Moreover, Microsporidia with horizontal transmission can increase female-biased sex ratio, either by feminization or male killing (Kelly et al. 2002; Terry et al. 2004; Green-Etxabe et al. 2015).

To study the potential impact of microsporidiosis on the behaviour and mutual interactions among the Ponto-Caspian amphipods, we focused on two model species: *Dikerogammarus villosus* (Sowinsky, 1894) and *Dikerogammarus haemobaphes* (Eichwald, 1841), considered as successful invasive alien species in Europe (Rewicz et al. 2015; Jażdżewska et al. 2020). They are widespread across Europe and co-occur in many places, sharing similar habitat preferences and life history traits (Grabows-ki et al. 2007; Bovy et al. 2015; Clinton et al. 2018). Usually *D. villosus* dominates and displaces *D. haemobaphes* (Kley and Mayer 2003; Bollache et al. 2004; Gruszka and Woźniczka 2008; Kinzler et al. 2009; Žganec et al. 2009; Bącela-Spychalska et al. 2012), though opposite situations have also been reported from the UK, where *D. haemobaphes* is more widely distributed (Clinton et al. 2018).

Several microsporidian parasites were identified to often infect these two model host species, both in native and colonised ranges: *Cucumispora dikerogammari* (Ovcharenko et al. 2010; Bącela-Spychalska et al. 2012, 2014), *Dictyocoela duebenum, D. berillonum, D. muelleri* (Wattier et al. 2007; Grabner et al. 2015; Green-Etxabe et al. 2015; Bącela-Spychalska et al. 2018) and *C. ornata* recorded from *D. haemobaphes* (Bojko et al. 2015, 2018). Some more Microsporidia are known to infect these hosts only from single records, e.g. *Nosema granulosis* (Wattier et al. 2007), thus these can be considered as accidental infections. These parasites differ in their impact on hosts, however such data are not available for all the species. *Cucumispora dikerogammari*, infecting both model *Dikerogammarus* species, has successfully spread to most European waters with its hosts (Wattier et al. 2007; Ovcharenko et al. 2010). This parasite, having a direct

life cycle, is considered as a highly virulent parasite, transmitted mostly horizontally through consumption and causing behavioural changes and mortality (Ovcharenko et al. 2010; Bącela-Spychalska et al. 2012, 2014). Nevertheless, the effect of parasitic Microsporidia on the functioning of amphipod assemblages has been still understudied.

We tested experimentally how the presence of parasitic *Cucumispora dikerogammari* modulates shelter competition between the two invasive amphipod species. We hypothesized that: (1) Amphipod behaviour would depend on (a) species (irrespectively of infection status) and (b) infection status; (2) Infection would affect intraspecific interactions among amphipods by weakening infected conspecifics (as being in a worse physical condition); (3) Non-infected individuals would avoid aggression towards and/ or contacts with infected specimens to reduce the risk of infection, as the parasite is mainly horizontally transmitted, through biting or consumption of infected tissue (Ovcharenko et al. 2010); (4) Amphipods would respond differently to conspecifics and heterospecifics; (5) Infection would affect interspecific interactions among amphipods by reducing the impact of infected individuals on their opponents (due to the mechanisms postulated in H2–3).

Materials and methods

Test organisms

We sampled D. villosus using artificial substratum traps in the Włocławek Dam Reservoir located in the lower Vistula River (N52.617738, E19.326453) and D. haemobaphes with benthic hand nets in the middle part of the Vistula River near the town of Połaniec (N50.423014, E21.311748) during the last week of May 2018. We transported the animals to the laboratory in plastic buckets with aerated water, placed in Styrofoam boxes filled with ice packs. We kept them in plastic containers $(40 \times 60 \times 12.5 \text{ cm}, \text{L} \times \text{W} \times \text{H})$ with gravel substratum (grain size 2–5 cm) at their average natural densities (c.a. 400 ind. m⁻²) (Dedju 1967). We used conditioned tap water, air-conditioning (17 °C) and light:dark cycle of 16:8 (which corresponded to the conditions at the sampling sites), and fed the amphipods daily with living chironomid larvae (commercially purchased) and dry fish food pellets. Every 3 days, we exchanged 30% of water. After one week of acclimatization, we used them in experiments. To avoid potential differences in aggression level between sexes as well as reproductive rather than aggressive relationships in conspecific pairs, we used only males in our experiments, distinguishing them by the presence of dense and long setation on the flagellum of the antenna II and large gnathopods (Eggers and Martens 2001). For each individual, we identified the species before the experiment by morphological features, such as the setation of the flagellum of the antenna II and the shape of the dorsal tubercles on the urosome segments I and II (Eggers and Martens 2001; Konopacka 2004). These two features are easy to observe and allow to discriminate between males of the two species by eye.

The very late stage of microsporidiosis is manifested by the whitish colour of the infected tissue (muscles), visible through the host cuticle even by eye (Ovcharenko et al. 2010). Symptomatic individuals can be in the lethal phase, likely to die in a few days (Bacela-Spychalska et al. 2012). Therefore, in the experiments, we used infected individuals before they started to exhibit any external symptoms of infection. Hence, we were able to detect and identify microsporidian infections only using molecular methods possible to apply after the experiments (see "Detection and identification of microsporidian parasites"). For the experiment, we used animals with natural infections acquired in the field, which reflected the situation in the environment. After the molecular diagnosis, we determined that *D. villosus* had been infected by a single Microsporidium species: *C. dikerogammari*, whereas *D. haemobaphes* was the host for three species: *C. dikerogammari*, *Dictyocoela berillonum* and *D. muelleri*. Due to insufficient numbers of all pairwise combinations of infection types, we only used non-infected amphipods and those infected with *C. dikerogammari* for our study to obtain a balanced design and sufficient number of replicates (Suppl. material 1: Table S1).

Experimental protocol

We performed experiments in glass dishes (diameter: 90 mm, height: 45 mm). A 20-mm high Plexiglas disk of the same diameter as the dish was put on its bottom. A hole (diameter: 7 mm, depth: 17 mm) was drilled in the disk 3 mm from its edge (Suppl. material 1: Fig. S1) to form a single shelter for amphipods. Such shelters were evidenced as suitable for amphipods, including *D. villosus*, by Platvoet et al. (2009b). We sealed gaps between the disk edges and glass walls of the dish with white plasticine to prevent amphipods from entering this space. The plasticine was proven as non-toxic for amphipods in our preliminary trials.

We aimed at testing shelter competition in all possible species vs. infection status combinations. We preliminarily screened both amphipod populations for the prevalence of various microsporidian species (see: "Detection and identification of microsporidian parasites" section), based on 100 individuals of each host species. This allowed us to roughly estimate the number of pairs to be tested to obtain sufficient numbers of all combinations. Altogether, we tested 80 conspecific pairs of *D. haemobaphes*, 219 conspecific pairs of *D. villosus* and 254 heterospecific pairs (Suppl. material 1: Table S1). We selected male individuals randomly with regard to their size.

We placed a pair of amphipods, both individuals marked with correction fluid to identify them during the analysis (the fluid and the marking procedure were proven as harmless during our preliminary trials), into an experimental dish, allowed them for 5-min acclimatization and recorded their behaviour for the next 30 min using a video camera (SNB-6004, Samsung, South Korea) located above the experimental arena. Water temperature was the same as in the stock tanks. Water was oxygenated before the test, thus, given its short duration, we assume that oxygen was not a limiting factor for the amphipods. After the test, we dried amphipods with a paper towel for 30 s to get rid of excess water (as described by Pöckl 1992) and weighed them to the nearest

0.01 g using a Kern microbalance (type PEJ, Germany) (wet weight) to assess their size. Finally, after the experiment, we fixed them individually in 96% ethanol to conduct molecular screening for the microsporidian presence and identification.

After molecular determination of microsporidian presence in each individual (see: "Detection and identification of microsporidian parasites" section), processed after the experimental trials, we were able to assign particular previously tested amphipod pairs to specific experimental treatments regarding their infection status (see Suppl. material 1: Table S1 for details).

Detection and identification of microsporidian parasites

We dissected muscle tissues from individual amphipods stored in 96% ethanol with forceps and incubated them at 55 °C in 1.5-ml tubes containing 200 µl of Queen's lysis buffer with 5 µl of proteinase K (20 mg ml⁻¹) according to the procedure by Seutin et al. (1991). We extracted total DNA (including microsporidian DNA, if present) using the standard phenol/chloroform method by Hillis et al. (1996) and resuspended dried DNA in 100 µl of TE buffer at pH 8 and stored at 4 °C until amplification. We conducted the PCR and used a pair of microsporidia-specific primers V1f/530r, following Baker et al. (1994) and Vossbrinck et al. (1993) to amplify distinctive parasite DNA fragments. We ran PCR reactions in 10 µl of reaction mixtures with each primer concentration of 400 nM, 200 μ M dNTPs and 0.5 U/ μ l Taq polymerase (Thermo Scientific). The product was amplified under the following PCR conditions: an initial denaturing step at 95 °C for 2 min was followed by 35 cycles of 95 °C for 30 s, 62 °C for 45 s and 72 °C for 1 min. These cycles were followed by a final extension at 72 °C for 5 min. We included a negative control containing no DNA and a positive control containing the known Microsporidium species in each set of PCR reactions. We visualised The PCR product on 2% agarose gel in order to identify positives - the presence of microsporidian DNA. Afterwards, we purified the selected positives with exonuclease I (Burlington, Canada) and FastAP alkaline phosphatase (Fermentas, Waltham, MA, USA) treatment and sequenced them directly with the BigDye technology by Macrogen Inc., (Amsterdam, The Netherlands) using the above-mentioned primers. We edited the obtained microsporidian sequences using Geneious R10 (http://www.geneious.com, Kearse et al. 2012). Then, we conducted the identification of microsporidia using BLAST in GenBank (https://www.ncbi.nlm.nih.gov/genbank).

Data analysis

We watched all the video recordings of amphipod behaviour manually (always the same person, to avoid bias) to determine: (1) Time spent in shelter by each individual, (2) Counts of aggression acts exhibited by each individual, when an amphipod touched the other individual with its antennae I and attempted to catch it with its gnathopods and antennae II (described as a sign of aggression by Platvoet et al. 2009b),

and (3) Time spent together in shelter by both individuals from the pair. Time variables were expressed as percentages of the total experimental time.

To test our hypotheses, we conducted four sets of General Linear Models (for time-related variables) and Generalized Linear Models with Poisson distribution and log link function (for aggression counts) using various subsets of the entire dataset (summarised in Suppl. material 1: Table S2). The division of the dataset was necessary due to the nature of the data, as explained below. The full dataset with subsets used for particular analyses described below is available in Suppl. material 2.

(1) To analyse intraspecific relationships among amphipods, we tested non-infected and infected individuals accompanied by non-infected and infected conspecifics. We had to divide this analysis into four separate models (Suppl. material 1: Table S2A-D), as the dataset followed partly a within-subject design (two members of a mixed pair consisting of an infected and non-infected individual were exposed together) and partly a between-subject design (selected members of uniformly infected and noninfected pairs were exposed in separate runs). Thus, to check the effect of the animals' own infection on their responses to conspecifics, we tested separately: (i) non-infected and infected responding amphipods accompanied by non-infected conspecifics (Suppl. material 1: Table S2A) and (ii) non-infected and infected responding amphipods accompanied by infected conspecifics (Suppl. material 1: Table S2B), using models including: (i) species and (ii) infection status of the responding individual. Moreover, to check the effect of the accompanying conspecific infection, we tested separately: (i) non-infected responding amphipods accompanied by non-infected or infected conspecifics (Suppl. material 1: Table S2C) and (ii) infected responding amphipods accompanied by non-infected or infected conspecifics (Suppl. material 1: Table S2D), using models including: (i) species and (ii) infection status of the accompanying individual.

(2) To check whether amphipods responded differently to individuals of various species, we compared the behaviour of non-infected and infected amphipods in the presence of conspecifics and heterospecifics (for simplification: non-infected only). Separate models were conducted for each amphipod species (Suppl. material 1: Table S2E-F), as conspecific/heterospecific identity was not the same for *D. villosus* and *D. haemobaphes*. The models included (i) infection status of the responding individual and (ii) accompanying species (conspecific or heterospecific).

(3) To check the effect of infection status on interspecific interactions among amphipods, we tested heterospecific pairs differing in infection status. The model (Suppl. material 1: Table S2G) included: (i) species of the responding individual (a within-subject factor, as both individuals of a heterospecific pair were included), (ii) infection status of the responding individual and (iii) infection status of the accompanying individual.

Using the above-mentioned models 1–3, we tested two response variables: time spent by responding amphipods in shelter and number of their aggression acts. Moreover, to further check the effect of infection status on intra- and interspecific interactions among amphipods we compared:

(4) The time spent together in the shelter by both individuals of the pair, using a model (Suppl. material 1: Table S2H) with treatment effect (all available combinations of species and infection status).

We selected responding animals from uniform pairs (conspecifics of the same infection status) randomly for the analyses. To control for the difference between masses of pair members, likely to affect the competition, we included a mass ratio (responding/accompanying individual) as a continuous predictor in models 1–2 above. In model 3, we included individual masses of both amphipods from each heterospecific pair as a continuous predictor. In model 4, we controlled for the effect of mass by including a mass ratio (larger/smaller individual) as a continuous predictor. We log-transformed the time-related variables prior to the analysis to meet General Linear Model conditions (normality tested with a Shapiro-Wilk test, homoscedasticity tested with a Levene test). As needed (i.e. when significant effects had more than 2 levels), we used sequential Bonferroni corrected pairwise LSD Fisher tests (General Linear Models) or pairwise contrasts (Generalized Linear Models) for posthoc comparisons. We conducted all statistical analyses using SPSS 27.0 statistical package (IBM Inc.).

Results

Intraspecific interactions among amphipods

Differences between the species. The only interspecific difference in shelter occupancy was the longer time spent in shelter by *D. villosus* compared to *D. haemobaphes* exposed to non-infected conspecifics (Fig. 1A, a significant species effect in Table 1A). Moreover, intraspecific aggression of *D. villosus* was always higher than that of *D. haemobaphes* (Fig. 1B, significant species effects in Table 1A–D).

Effect of the infection status of the responding individual. The infection status did not affect time spent by amphipods in shelter and their aggression in the presence of noninfected conspecifics (non-significant infection effects for both behaviours in Table 1A). On the other hand, in the presence of infected conspecifics, non-infected amphipods of both species occupied shelters for a longer time (Fig. 1A) and were less aggressive (Fig. 1B) than infected individuals (significant infection effects for both behaviours in Table 1B).

Effect of the infection status of the accompanying conspecific. Individuals of both species, irrespective of their own infection status, spent more time in shelter in the presence of infected rather than non-infected conspecifics (Fig. 1A), as shown by significant infection effects in Table 1C, D). Non-infected amphipods of both species were more aggressive towards non-infected than towards infected conspecifics (Fig. 1B, a significant infection effect in Table1C), whereas intraspecific aggression of infected amphipods was unrelated to the infection status of accompanying conspecifics (Fig. 1B, Table 1D).

| Table I. Analyses of the effect of infection and species identity on intra- and interspecific interaction |
|--|
| among amphipods. We analysed shelter occupancy time and number of aggression acts with the Genera |
| and Generalized Linear Models (Poisson distribution, log link function), respectively. |

| Analysis | Effect | df | Time in shelter | | Aggression | |
|---|------------------------|--------|-----------------|----------|------------|-------------|
| | | | F | Р | F | Р |
| A. Responses of infected vs. non- | Species1 | 1,210 | 6.63 | 0.011* | 76.23 | < 0.001* |
| infected amphipods to non- | Infection1 | 1,210 | 2.50 | 0.115 | 0.01 | 0.935 |
| infected conspecifics | Sp1*Inf1 | 1,210 | 2.53 | 0.113 | 0.03 | 0.855 |
| | Mass ratio | 1,210 | 0.25 | 0.618 | 5.71 | 0.018^{*} |
| B. Responses of infected vs. non- | Species1 | 1,177 | 0.66 | 0.417 | 17.94 | < 0.001* |
| infected amphipods to infected conspecifics | Infection1 | 1,177 | 8.66 | 0.004* | 23.21 | < 0.001* |
| | Sp1*Inf1 | 1,177 | 0.25 | 0.620 | 1.50 | 0.222 |
| | Mass ratio | 1,177 | 11.21 | 0.001 | 6.59 | 0.011* |
| C. Responses of non-infected | Species1 | 1,210 | 0.16 | 0.692 | 19.79 | < 0.001* |
| amphipods to infected vs. non- infected conspecifics | Infection2 | 1,210 | 19.00 | < 0.001* | 14.63 | < 0.001* |
| | Sp1*Inf2 | 1,210 | 1.50 | 0.223 | 0.03 | 0.865 |
| | Mass ratio | 1,210 | 3.97 | 0.048 | 7.95 | 0.005* |
| D. Responses of infected amphipods | Species1 | 1,177 | 1.99 | 0.160 | 39.39 | < 0.001* |
| to infected vs. non-infected | Infection2 | 1,177 | 5.40 | 0.021* | 0.02 | 0.890 |
| conspecifics | Sp1*Inf2 | 1,177 | 2.91 | 0.090 | 3.50 | 0.063 |
| | Mass ratio | 1,177 | 3.31 | 0.070 | 1.64 | 0.202 |
| E. Responses of infected vs. non- | Species2 | 1, 338 | 1.80 | 0.181 | 0.17 | 0.677 |
| infected <i>D. haemobaphes</i> to non-infected conspecifics vs. | Infection1 | 1, 338 | 1.63 | 0.202 | 1.54 | 0.215 |
| | Sp2*Inf1 | 1, 338 | 1.08 | 0.300 | 0.65 | 0.420 |
| heterospecifics | Mass ratio | 1, 338 | 0.12 | 0.728 | 19.31 | < 0.001* |
| F. Responses of infected vs. | Species2 | 1, 338 | 0.44 | 0.510 | 32.28 | < 0.001* |
| non-infected D. villosus to | Infection1 | 1, 338 | 1.32 | 0.251 | 2.85 | 0.092 |
| non-infected conspecifics vs. | Sp2*Inf1 | 1, 338 | 1.33 | 0.249 | 1.98 | 0.160 |
| heterospecifics | Mass ratio | 1, 338 | 4.99 | 0.026 | 4.70 | 0.031* |
| G. Responses of infected vs. non- | Species1 ^{ws} | 1, 499 | 4.55 | 0.033* | 5.48 | 0.020* |
| infected amphipods to infected | Infection1 | 1, 499 | 1.37 | 0.243 | 2.19 | 0.140 |
| vs. non-infected heterospecifics | Infection2 | 1, 499 | 0.86 | 0.356 | 0.05 | 0.830 |
| | Sp1*Inf1 | 1, 499 | 1.92 | 0.166 | 0.04 | 0.847 |
| | Sp1*Inf2 | 1, 499 | 5.91 | 0.015* | 0.07 | 0.792 |
| | Inf1*Inf2 | 1, 499 | 0.09 | 0.761 | 0.11 | 0.744 |
| | Sp1*Inf1*Inf2 | 1, 499 | 0.09 | 0.771 | 0.21 | 0.650 |
| | Mass | 1, 499 | 0.46 | 0.498 | 3.62 | 0.058 |
| H. Time spent together in the shelter | Pair comp. | 9, 542 | 4.25 | < 0.001* | | |
| | Mass ratio | 1, 542 | 0.45 | 0.504 | | |

Species1/2 (Sp1/Sp2) - species of the responding / accompanying individual, respectively (D. haemobaphes or D. villosus)

Infection1/2 (Inf1/Inf2) - infection status of the responding / accompanying individual, respectively (infected or non-infected)

Pair comp. – species composition and infection status of the amphipod pair in analysis H: both *D. haemobaphes*, both *D. villosus* or heterospecific × both infected, infected & non-infected or both non-infected

Mass ratio - responding / accompanying individual (analyses A-F) or larger / smaller individual (analysis H)

Mass – individual mass (analysis G)

WS - within-subject factor (analysis G)

Responding individuals from uniform pairs (conspecifics with the same infection status) in analyses A-F were randomly selected from the pair

Differences between intra- and interspecific interactions

Dikerogammarus haemobaphes did not change its shelter occupancy time and aggression depending on the species identity of the accompanying individual (Fig. 2, non-significant species effects for both behaviours in Table 1E). On the other hand, *D. villosus* was more aggressive towards conspecifics than towards *D. haemobaphes* (Fig. 2B, Table 1F), though its shelter occupancy time was unaffected by the accompanying species identity (Table 1F).

Interspecific interactions among amphipods

In the presence of *D. villosus*, *D. haemobaphes* spent more time in shelter when the accompanying individual was infected rather than non-infected (Fig. 3A). On the other hand, shelter occupancy time of *D. villosus* did not depend on the infection status of the accompanying *D. haemobaphes*, which resulted in a significant species*accompanying individual infection interaction (Table 1G). Shelter occupancy time by amphipods was independent of the infection status of the responding individual (non-significant effects involving the responding individual infection in Table 1G).

Interspecific aggression of *D. villosus* was higher than that of *D. haemobaphes* (Fig. 3B, a significant species effect in Table 1G), irrespective of the infection status of responding or accompanying individuals (non-significant infection effects in Table 1G).

Time spent by amphipod pairs together in shelter

Time spent together by both individuals in shelter depended on pair composition (Table 1H). Non-infected heterospecific pairs spent more time together in shelter than non-infected conspecific pairs (Fig. 4). Moreover, infected heterospecific and *D. villosus* pairs spent more time together in shelter than infected *D. haemobaphes* pairs. Furthermore, non-infected *D. villosus* pairs spent less time together in shelter than *D. villosus* pairs with at least one infected individual (Fig. 4).

Discussion

Interspecific differences

As predicted by hypothesis 1a, both species differed from each other in behaviour. Interspecific differences in shelter occupancy time were inconsistent. *Dikerogammarus haemobaphes* spent more time in the shelter than *D. villosus* when exposed to infected heterospecifics (Fig. 3A), whereas an opposite difference occurred between individuals of these species exposed to non-infected conspecifics (Fig. 1A). This suggests that shelter occupancy depended more on the identity of the accompanying individual (both species spent more time in shelter in the presence of accompanying *D. villosus*) than on the responding amphipod. Nevertheless, both intra- and interspecific aggression (Fig. 1B and Fig. 3B, respectively) of *D. villosus* was consistently higher than that of *D. haemobaphes*. The former species is often considered as the strongest competitor of all invasive Ponto-Caspian amphipods, aggressively displacing native and alien relatives (Dick and Platvoet 2000; Krisp and Maier 2005), including *D. haemobaphes* (Kley and Maier 2003; Žganec et al. 2009), from novel areas. Its high aggression level, enabling efficient interference competition, is congruent with high invasiveness (van Riel et al. 2009; Bertelsmeier et al. 2015; Grether et al. 2017). Both these species are



Figure 1. Effect of infection on intraspecific interactions among amphipods. Shelter occupancy time **A** and number of aggression acts **B** shown by infected (black) or non-infected (white) *D. haemobaphes* (circles) and *D. villosus* (squares) in response to infected (red border) or non-infected (blue border) conspecifics. Results are back-transformed least squares means (\pm 95% confidence intervals) predicted for significant effects by the General or Generalized Linear Models (analyses A–D in Table 1 and Suppl. material 1: Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another.

typical "sit and wait" organisms exhibiting low activity and spread rate (Platvoet et al. 2009a; Beggel et al. 2016), which is especially true for *D. haemobaphes* not exposed to interspecific competition (Kobak et al. 2016; Rachalewski et al. 2019).

Intraspecific interactions among amphipods

In conspecific pairs, the highest shelter occupancy time was exhibited by non-infected amphipods exposed to infected conspecifics (Fig. 1A). Thus the infection of the accompanying individual resulted in the higher shelter occupancy time, especially when the responding individual was not infected. Moreover, non-infected amphipods were less aggressive towards infected vs. non-infected conspecifics, confirming our hypothesis 3, whereas no such difference was exhibited by infected animals (Fig. 1B).

The increased shelter occupancy time in the presence of infected conspecifics indicates that infected individuals posed a lower competitive pressure. They were either more easily displaced from the shelter or allowed their competitors to occupy the shelter together with them. The fact that the amount of time spent together by both *D. villosus* individuals in the shelter increased when at least one of them was infected (Fig. 4) supports the latter explanation. The aggression of infected individuals was not reduced (Fig. 1B), thus it is likely that they were less efficient in their attempts to seize the shelter than non-infected conspecifics, e.g. due to weaker condition or locomotor ability.

The reduced aggression of non-infected amphipods towards infected vs. infected conspecifics (Fig. 1B), in line with unchanged aggression level of infected individuals, suggest that the reduction in intraspecific aggression depended on the infection status of the attacked individual, rather than on that of the attacker. In other words, amphipods avoided to attack infected conspecifics (though did not avoid their company in the shelter). This may be accounted for by an attempt to reduce the probability of infection. *Cucumispora dikerogammari* is transmitted horizontally (by consumption, thus biting may be dangerous) and causes a lethal disease in their hosts (Bącela-Spychalska et al. 2012). Recognition of infected conspecifics and refraining from dangerous contacts with them is a widespread mechanism of infection avoidance in animals (Curtis 2014; Øverli and Johansen 2019). This shows that the effects of parasites on their hosts may be sometimes quite subtle, not manifested by direct changes in survival or appearance, but exhibited in specific situations, such as the high competitive pressure (MacNeil et al. 2003).

On the other hand, infected amphipods of both species did not diversify their responses depending on the infection status of their opponent (Fig. 1B). Thus, infection is likely to disrupt natural behaviour of amphipods, which may reduce their ability to respond appropriately to environmental factors.

To summarize, in accordance with our hypothesis 2, Microsporidia reduced competitive abilities of both amphipod hosts: infected individuals performed worse in shelter competition against their non-infected conspecifics.

Interspecific interactions among amphipods

Amphipod shelter occupancy time did not depend on the accompanying species identity (Fig. 2A), but, in accordance with our hypothesis 4, *D. villosus* was more aggressive towards conspecifics than towards *D. haemobaphes* (Fig. 2B). In contrast, the intra- and interspecific aggression levels of *D. haemobaphes* were similar to each other (Fig. 2B) and consistently lower than those of *D. villosus* (Fig. 3B). On the other hand, heterospecific pairs spent more time together in shelter than conspecific pairs of both amphipod species, suggesting the higher level of negative intraspecific relationships also in *D. haemobaphes* (Fig. 4). The higher intraspecific aggression shown by *D. villosus* in our study, is a common situation in the nature (Connell 1983). Conspecifics use the same resources and therefore pose a stronger competitive pressure than heterospecifics, even from the same guild. On the other hand, strong interspecific aggression was also indirectly observed in another pair of freshwater Ponto-Caspian amphipods, *D. villosus* and *Pontogammarus robustoides* (Jermacz et al. 2015) and many examples of strong interspecific interferences can be found in nature (Amarasekare 2002).

Surprisingly, *D. villosus* did not affect the shelter occupancy of *D. haemobaphes* more than conspecifics did (Fig. 2A). *Dikerogammarus haemobaphes* showed relatively low level of aggression towards both species. When given such a possibility, it usually migrates away from sites occupied by *D. villosus* (Kobak et al. 2016) and actively escapes from its scent (Rachalewski et al. 2019). Thus, *D. haemobaphes* seems to avoid direct encounters



Figure 2. Amphipod responses to conspecific and heterospecific opponents **A** shelter occupancy time and **B** number of aggression acts shown by *D. haemobaphes* (circles) and *D. villosus* (squares) (pooled infection status) in response to non-infected conspecifics and heterospecifics. Results are back-transformed least squares means (±95% confidence intervals) predicted for significant effects by the General or Generalized Linear Models (analyses E-F in Table 1 and Suppl. material 1, Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another. Non-significant effects are pooled.



Figure 3. Effect of infection on interspecific interactions among amphipods **A** shelter occupancy time and **B** number of aggression acts shown by *D. haemobaphes* (circles) and *D. villosus* (squares) (pooled infection status) in response to heterospecifics of various infection status: infected (red border), noninfected (blue border) or pooled (grey border). Results are back-transformed least squares means ($\pm 95\%$ confidence intervals) predicted for significant effects by the General or Generalized Linear Model (analysis G in Table 1 and Suppl. material 1, Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another. Non-significant effects are pooled.

with *D. villosus* in the environment. That is why *D. villosus* might show lower aggression towards *D. haemobaphes* than towards conspecifics. The displacement between the two species, often observed in the field (Kley and Maier 2003), may depend on the active avoidance of *D. villosus* by *D. haemobaphes* rather than on direct aggression and interfer-



Figure 4. The effect of infection on time spent together in shelter by two amphipods. Pairs were composed of infected or non-infected *D. haemobaphes* and/or *D. villosus*. Results are back-transformed least squares means (±95% confidence intervals) predicted by the General Linear Model (analysis H in Table 1 and Suppl. material 1: Table S2). Treatments marked with the same lowercase letters did not differ significantly from one another.

ence competition among them. The ability to assess their own chances and avoid a direct conflict with a stronger opponent allows animals to minimize their energy losses and risk of injures (Parker and Rubenstein 1981). In the wild, *D. haemobaphes* can retreat to habitats avoided by its stronger competitor, e.g. with stronger water flow (Borza et al. 2018). Anyway, our study shows that, when migration is not possible, *D. haemobaphes* is capable of withstanding the direct co-existence with *D. villosus* without any visible negative consequences in shelter use, at least over a short term tested in our study.

In accordance with our hypothesis 5, infection status did affect interspecific interactions among amphipods. Infected and non-infected amphipods did not differ from each other in their shelter occupancy time in the presence of heterospecific opponents, but the infection status of the opponent did affect the responses of *D. haemobaphes*: they spent more time in the shelter in the presence of infected rather than non-infected heterospecifics (Fig. 3A). Thus, similarly to intraspecific interactions, they utilized shelters more efficiently in the presence of infected *D. villosus*. It has been already evidenced that the presence of microsporidian and/or acanthocephalan parasites reduces the intraguild predation pressure among amphipod species and facilitates their coexistence (e.g. MacNeil and Dick 2011). In contrast to intraspecific interactions, amphipods did not change their aggression rate depending on the infection status of the accompanying heterospecific. Perhaps they are only able to recognize the infection in conspecific competitors, or the level of interspecific aggression is already so low that the danger of getting infected after biting an infected heterospecific competitor is negligible.

To summarize, according to our hypothesis 5, infection increased amphipod shelter occupancy in heterospecific dyads and thus could contribute to the co-existence of the two species over a longer time scale. Nevertheless, the effect of infection on interspecific relationships was less pronounced, particularly in terms of aggression changes, than in the case of intraspecific interactions.

Conclusions

In general, parasites tended to reduce the ability of their hosts to defend their shelters, though did not directly reduce their aggression. This indicates the reduced competitive abilities of infected amphipods and relatively improved performance of their noninfected opponents. However, in terms of shelter occupancy time, overall benefits of the non-infected individuals seem greater than losses of the infected animals, particularly given the fact that amphipods tended to reduce their aggression towards infected conspecifics. Dikerogammarus haemobaphes benefited (in terms of the longer shelter occupancy) from the presence of infected conspecifics and heterospecifics, whereas D. villosus increased its shelter occupancy only in response to infected conspecifics. Thus, parasites, apart from their apparent negative direct effects on their hosts, at the population and community levels may promote species co-existence rather than displacement. Obviously, confirmation of such a conclusion needs a longer-term study than our 30-min long experiment, but shelter use is an important life parameter of these sit-and-wait organisms, shaping their performance in the wild to a high extent. Although the Microsporidium species under our study causes a lethal disease, its presence may temporarily, before the terminal phase, result in locally increased population densities due to the lower levels of interference competition. This, in turn, may increase the impact of the amphipod assemblage on the local community. Given highly variable (both spatially and temporally) levels of Microsporidium prevalence in amphipod assemblages (Bacela-Spychalska et al. 2012), parasite presence may account for contrasting outcomes of their interspecific competition, leading to displacement or coexistence. If C. dikerogammari infection had been more virulent to D. villosus (a theoretically stronger competitor) than to D. haemobaphes, the parasite would have been likely to sustain their coexistence through apparent competition. Whether these mechanisms translate into cascading ecosystem level effects on other organisms, such as local predators, prey and competitors of invasive amphipod assemblages, remains to be determined in future studies. Nevertheless, parasitic infection seems to be a likely and so far overlooked factor contributing to discrepancies among various studies describing the effects of the Ponto-Caspian community on invaded communities. For

instance, *D. villosus* has been shown as an efficient carnivore (Dick et al. 2002; Krisp and Maier 2005; Bącela-Spychalska and Van der Velde 2013), occupying the niche corresponding to that of small fish (van Riel et al. 2006) and showing low activity as a shredder of coarse plant detritus (MacNeil et al. 2011), but also, in contrast to the above-mentioned statements, as an efficient shredder and herbivore with a low share of food of animal origin in its diet (Hellmann et al. 2015; Koester et al. 2016).

Our study shows that parasitic infections play an important role in shaping biological invasions not only by mediating interactions between invasive and local organisms, as it has been shown previously (Dunn 2009; Dunn et al. 2012; Dunn and Hatcher 2015; Hatcher et al. 2015), but also by affecting interactions among various invasive species and likely shaping their impact on the environment. The presence of Microsporidia in our model system may contribute to the invasional meltdown phenomenon (Simberloff and Von Holle 1999) by improving the coexistence of invasive species. Incorporating additional invasive species, in this case the parasites, makes the composition of the invasive host species assemblage more complex. Without microsporidians infecting the top competitor, the weaker species could be displaced, but, in the presence of these parasites, it may be able to face the infected opponent. As a result, the number of invasive species in the community (consisting of hosts and parasites) may be expected to be higher than in a parasite-free community. On the other hand, microsporidian parasites infecting D. villosus may diminish its predatory pressure and moderate its impact on local biota (as shown by Bacela-Spychalska et al. 2014), thus reducing its negative impact. This would be in contrast to another assumption of the invasional meltdown hypothesis, namely the exacerbated impact of invasive species on local biota due to the appearance of additional invaders (Simberloff and Von Holle 1999). Thus, the addition of alien parasites to the community may have contradictory effects on the appearance of the invasional meltdown phenomenon.

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Supplementary material I

Figure S1, Tables S1, S2

Authors: Jarosław Kobak, Michał Rachalewski, Karolina Bącela-Spychalska Data type: Pdf file

- Explanation note: Figure S1. Experimental setup. Table S1. Numbers of particular amphipod pairs obtained in the study. Table S2. Analyses carried out within the study.
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Supplementary material 2

Dataset

Authors: Jarosław Kobak, Michał Rachalewski, Karolina Bącela-Spychalska

Data type: excel file

Explanation note: Experimental data.

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LETTER TO THE EDITOR



Global costs of plant invasions must not be underestimated

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The impacts of biological invasions have become a key focus of researchers in recent decades, leading to a rapid accumulation of evidence on economic losses associated with invasions. In a synthesis paper, Diagne et al. (2021) use a new database, InvaCost (Diagne et al. 2020), to quantify the global economic costs of biological invasions. They demonstrate that the global costs associated with invasive alien species are massive, at least US\$ 1.3 trillion between 1970 and 2017, and increasing rapidly. Such high costs emphasize the critical importance of preventing and controlling biological invasions. Their paper thus delivers an important and much needed contribution to invasion science, which can strengthen invasive alien species management and policy globally. However, the costs of plant invasions presented by Diagne et al. (2021) are substantially underestimated compared to those of vertebrate and invertebrate invasions, and with respect to the available literature. While Diagne et al. (2021) state that the reported costs have pronounced geographic and taxonomic gaps, we believe that their significant underestimation of plant costs in comparison with other taxonomic groups needs to be clarified, to correctly demonstrate the severity of plant

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invasions and guide appropriate prioritization, budgeting, and allocation of limited management resources.

Diagne et al. (2021) report that invasive alien plants contribute 1.5% of the total costs that can be attributed to a single taxonomic group between 1970 and 2017 (i.e. plants cost US\$ 8.9 billion out of a total of US\$ 591 billion attributable to plants, vertebrates or invertebrates). They acknowledge that the low representation of invasive alien plants "is probably due to a data deficiency in the current database". Indeed, subsequent updates to the InvaCost database revealed the unbalanced distribution of data entries among taxonomic groups in the version of the database used by Diagne et al. (2021) (InvaCost v1; https://doi.org/10.6084/m9.figshare.12668570.v1), as compared to subsequent versions. In particular, while the full InvaCost v1 database contained 98 data sources (publications, reports, etc.) on the costs of plant invasions out of 786 sources across all taxonomic groups, the first update, released in November 2020, added 623 sources to the full database, of which the majority (416 sources) included plant costs (InvaCost v3, https://doi.org/10.6084/m9.figshare.12668570.v3). Furthermore, the most recent version of the InvaCost database (https://doi.org/10.6084/ m9.figshare.12668570.v4) shows that over 80% of currently available data sources on the costs of plant invasions were absent from InvaCost v1. In comparison, less than half of the available sources on animal invasions were absent from InvaCost v1. While we appreciate that databases evolve as new information becomes available, and we welcome and applaud such updates, these numbers indicate a substantial discrepancy in the compilation of data for plants vs. animals in InvaCost v1. It seems inevitable that these biases in survey effort affected the numbers and proportional costs attributed to plants by Diagne et al. (2021).

Research published over the last 20 years also clearly shows that the global costs of plant invasions are much higher than the US\$ 8.9 billion reported by Diagne et al. (2021). For example, the total estimated cost of invasive alien plants in the South African fynbos alone is estimated at US\$ 11.8 billion (van Wilgen et al. 2001). In Europe, invasive alien plants have been found to cost at least € 3.8 billion annually, accounting for 30% of total invasion costs in the continent (Kettunen et al. 2009). In another study, a single invasive alien plant, Ambrosia artemisiifolia (Asteraceae), has been reported to cost the European economy US\$ 4.5 billion annually (Bullock et al. 2012). This means that over four decades the cost of A. artemisiifolia would approximate that of the costliest taxa presented by Diagne et al. (2021), two mosquito species (Aedes aegypti and A. albopictus), which accounted for US\$ 148.7 billion between 1970 and 2017. Yet, no plant appears among the costliest taxa listed by Diagne et al. (2021). Similarly, the cost of invasive alien plants to the Australian economy within agricultural areas alone is estimated at US\$ 4 billion annually (Sinden et al. 2005), and aquatic invasive alien plants in 13 public lakes in Florida cause annual costs of US\$ 6 billion (Adams and Lee 2007). Plants also feature prominently among the world's "100 of the worst" invasive alien species (Boudjelas et al. 2000), of which Euphorbia esula

(Euphorbiaceae) ranks among the top ten economically most damaging species with a global cost of US\$ 7.3 billion between 1960 and 2020 (Cuthbert et al. 2021). This selection of studies alone – which is by no means exhaustive – illustrates that the real costs of plant invasions globally must be orders of magnitude higher than the US\$8.9 billion estimate reported by Diagne et al. (2021).

Available funding for effective invasive alien species management is generally scarce and resources need to be allocated efficiently and systematically (Dana et al. 2019). However, in reality budgets dedicated to environmental management are often prone to taxonomic bias (Mammola et al. 2020). By disproportionately underestimating plant costs, support for invasive alien plant management could be inappropriately deprioritized, which would have serious ecological and socioeconomic consequences. Continued updates to the InvaCost database will provide further clarity on the true known costs of invasive alien plants. However, as these examples illustrate, local and regional studies already provide strong evidence that invasive alien plants have caused substantial economic costs, which are comparable to those of invasive alien vertebrates or invertebrates. This important message needs to be highlighted to ensure that decisions associated with studying and managing biological invasions are based on the most accurate economic cost estimates, and resources are allocated on the basis of the best available science.

Therefore, we argue that the important message conveyed by Diagne et al. (2021) on the massive economic costs of biological invasions needs to be complemented to emphasize that (i) the costs of invasive alien plants are similar in magnitude to those incurred by invasive alien vertebrates or invertebrates; (ii) further work is needed to estimate and aggregate the costs of invasive alien plants worldwide; and (iii) preventing and controlling plant invasions should remain a key component of international, national and regional invasive alien species management and policy decisions.

Acknowledgements

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Pathogens co-transported with invasive non-native aquatic species: implications for risk analysis and legislation

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Abstract

Invasive Non-Native Species (INNS) can co-transport externally and internally other organisms including viruses, bacteria and other eukaryotes (including metazoan parasites), collectively referred to as the symbiome. These symbiotic organisms include pathogens, a small minority of which are subject to surveillance and regulatory control, but most of which are currently unscrutinized and/or unknown. These putatively pathogenetic symbionts can potentially pose diverse risks to other species, with implications for increased epidemiological risk to agriculture and aquaculture, wildlife/ecosystems, and human health (zoonotic diseases). The risks and impacts arising from co-transported known pathogens and other symbionts of unknown pathogenic virulence, remain largely unexplored, unlegislated, and difficult to identify and quantify. Here, we propose a workflow using PubMed and Google Scholar to systematically search existing literature to determine any known and potential pathogens of aquatic INNS. This workflow acts as a prerequisite for assessing the nature and risk posed by co-transported pathogens of INNS; of which a

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better understanding is necessary to inform policy and INNS risk assessments. Addressing this evidence gap will be instrumental to devise an appropriate set of statutory responsibilities with respect to these symbionts, and to underpin new and more effective legislative processes relating to the disease screening and risk assessment of INNS.

Keywords

Alien species, invasive pathogen, opportunistic pathogen, parasite, symbiont

Introduction

Invasive Non-Native Species (INNS) are "species whose introduction by human activity outside their natural past or present distribution threatens biodiversity", as defined by the Convention on Biological Diversity (CBD 2010), and are one of the greatest global threats to biodiversity (IPBES global assessment 2019). New introductions of INNS are increasing every year, with no indication that introduction events are decreasing in frequency (Seebens et al. 2020). It is increasingly recognized that invasions are not the product of single species introduction events but can be considered as holobionts (Skillings 2016): i.e., units of biological organisation including the host and all its symbionts (external and internal), including pathogenic species. Therefore, organisms such as viruses, bacteria, fungi, protists and other (micro-)eukaryotic parasites and pathogens may be introduced to new regions along with their invasive non-native host and can be important factors in the invasion process (Peeler et al. 2011; Roy et al. 2017). A broad basis for referring to these organisms as 'pathogens' is required: they may not be recognized as pathogens, or cause disease in the INNS host transporting them, but may impact on other related (or unrelated) hosts in their new range. Further, pathogenesis can be very context dependent, as described by the symbiotic continuum concept (Bass and del Campo 2020). Therefore, a biologically informed approach to horizon scanning for such 'pathogens' is necessary, to enable effective identification of potentially new and emerging diseases. For the purposes of this paper, to avoid repetition of "parasites/pathogens" to refer to symbionts that take nutritional advantage of their hosts potentially causing disease, we henceforth use "pathogen" as a catch-all term.

In the field of invasion biology, the translocation of non-native pathogens (emerging infectious diseases in public and wildlife health) are increasingly being researched as important environmental driving factors (Ogden et al. 2019; Thakur et al. 2019); however, this is not currently reflected in national and international policy and legislation. For example, co-introduced pathogens are explicitly excluded from the EU Invasive Alien Species Regulation 1143/2014. Instead, potentially invasive co-introduced pathogens are considered as potential impacts of INNS establishment. Although pathogens are currently excluded from much of the legislation surrounding invasive species, there are a few examples where they are included, for example the Ballast Water Management Convention (Hess-Erga et al. 2019). Understanding and predicting the impacts of INNS is essential to inform risk analysis, for example, via horizon scanning, risk assessments and impact assessments, which underpin many components of INNS policy and management. However, pathogens associated with most (potential) INNS are very poorly known (Roy et al. 2017; Pagenkopp Lohan et al. 2020), except in the few cases where they are recognized under animal disease or human health legislation and are monitored and reported accordingly. In general, INNS risk analyses focus on the environmental and/or cumulative impacts of INNS, without (specific) reference to co-transported pathogens (e.g., Dick et al. 2017).

Knowledge and policy gaps can result in inadequate scrutiny and assessment of the risks associated with the movement of pathogens into new regions and countries (Hulme 2014; Dunn and Hatcher 2015). This has been recognized with a call for the prioritization of empirical research required to cover knowledge gaps about transmitted pathogens (Chinchio et al. 2020). Therefore, a framework for quantifying and documenting our existing knowledge of INNS and associated pathogens which may also become introduced with host movements is vital. This involves conducting literaturebased and pathogen screening to fill knowledge gaps where such information is lacking. These data will then lead to the development of invader pathogen profiles, outlining what is known about the invader's pathogens and those of related taxa. A complexity in this process is the diversity within the pathogen profile of a given species across its invasive range (e.g., Bojko et al. 2018), where any single INNS may have multiple different disease profiles across its native and invasive range, which will also change over time. This potential spatial and temporal variation in the pathogen profile of a given species could potentially drive the need for more specific risk assessments in relation to invasion risks (i.e., not only a particular species, but also from a particular population).

In this paper we present a workflow to meet these imperatives. This can be applied to INNS already present in a region, those with the potential to arrive, and those already present but yet to establish. For the purposes of this paper, we focus on (potentially) pathogenic symbionts of aquatic INNS of concern to the UK, which may be permanently or transiently associated with one or multiple water bodies. We include all pathogenic symbiont types: viral, microscopic, and macroscopic parasites (including metazoans). The underlying premise can be applied across all habitat types, and all symbionts including pathogens that manipulate behaviors of one or more of their hosts, and symbionts that have no discernible effect on their hosts.

Biology and ecology of pathogens co-transported with INNS

The movement of INNS beyond their native range can result in changes to established host-pathogen relationships, including INNS losing or gaining parasites (Peeler et al. 2011; Dunn and Hatcher 2015; Vilcinskas 2015). The multitude of potential outcomes resulting from relationship changes are summarized in the schematic shown in Figure 1. The enemy release hypothesis (see glossary) states that INNS can lose their pathogens as they move into a new range, which may be due to ecological factors, or



Figure 1. Potential fates of symbionts (including pathogens) co-transported with INN host species. The left-hand panel represents a hypothetical INNS with a symbiome comprising pathogens **A**, **B** and uncharacterized symbionts **1–3**. Potential symbionts already in the native system are pathogens **C– F** and uncharacterized symbionts **4–6**. Symbionts can be gained and/or lost by INNS hosts. The main panel on the right presents, with examples, scenarios of gains, losses, and transfers between non-native and native hosts of different species, and outcomes associated with such interactions. Skull and crossbones indicates death/negative effects to native host population. Boxes with gray fill indicate theoretical outcomes for which no empirical evidence was found.

for heteroecious parasites, the absence of a secondary host (Colautti et al. 2004). Cointroduced pathogens can potentially infect native species (Keane and Crawley 2002). In some cases, pathogen loss can also increase invasion success by reducing pathogen burden and associated health costs as well as reducing/eliminating competing susceptible native species (Prenter et al. 2014). Furthermore, lack of co-evolution potentially results in the increased susceptibility of native hosts to the invading pathogen (Taraschewski 2006). For example, a study comparing invasive pathogenicity in cointroduced and native hosts suggests that in 85% of cases it is higher in native hosts compared to non-native hosts (Lymbery et al. 2014).

Co-introduced pathogens can have significant effects on both native and invasive host evolution, and also different populations of the same host species (Blakeslee et al. 2019a). For example, *Rhithropanopeus harrisii* has adapted to parasitism by an introduced castrating rhizocephalan parasite, *Loxothylacus panopaei*, resulting in much higher pathogen prevalence in its introduced range where the host is naive. This demonstrates the potential consequences of parasite introduction and transmission host populations where they lack an evolutionary relationship (Tepolt et al. 2019). Cointroduced pathogens can also suffer genetic founder effects themselves; this is particularly exhibited in obligate parasitic organisms with complex life cycles. Trematodes infecting the invasive eastern mudsnail (*Tritia obsoleta*) have been shown to have significantly lower genetic diversity in their introduced range compared to their native range (Blakeslee et al. 2019b).

INNS can also affect native host-pathogen relationships, altering population dynamics and disease transmission. Thieltges et al. (2008) demonstrated that the presence of invasive *Crepidula fornicata* and *Crassostrea gigas* significantly reduced the trematode parasite burden of native *Mytilis edulis*, by interfering with the transmission of freeliving infective trematode larval stages and therefore reducing infection of *M. edulis*. Host-pathogen ecosystem interactions prove complex, creating challenges for the prediction of invasion success at different locations. The invasive amphipod *Echinogammarus ischnus* has outcompeted the native *Gammarus fasciatus* at many locations in the Great Lakes and St. Lawrence River in North America through predation and competition (Dermott et al. 1998). However, a native oomycete infects the invasive *E. ischnus* and causes greater mortality to the invasive host than to the native *G. fasciatus*, which facilitates the coexistence of the two species in areas of disease prevalence (Kestrup et al. 2011). This relationship is further nuanced in that *E. ishnus* can also act as a reservoir of the oomycete and facilitate parasite spillback to native amphipods.

Many diseases initially thought to be caused by one primary agent are now known to be the result of interactions between multiple symbionts, the host, and their environment; resulting in the pathobiome concept (Bass et al. 2019). Each INNS individually co-transports its own symbiome, making it difficult to predict its effect on the invaded ecosystem. A survey of symbionts of the invasive green crab *Carcinus maenas* in its native and invaded range showed many co-transported parasites persisted within the host at its invasion territory (Bojko et al. 2018). The latest approximation suggests this species is associated with ~82 known symbionts, many of which are pathogenic and pose risks to native ecosystems and the bioeconomy (Bojko et al. 2020).

The combination of hosts and their symbionts is of more immediate concern than considering the simple transposition of a pathogenic agent, such as a single virus or bacterium. Co-introduction of symbionts with an INNS is more likely to result in pathogen establishment because the co-evolved biological system is already in place to facilitate transmission (Peeler et al. 2011). Generalist pathogens are the main cause for concern since they can utilize native hosts more readily (Peeler et al. 2011). Symbiotic co-invaders may also present parasitic traits in new locations. For example, *Aphanomyces astaci*, the oomycete agent of crayfish plague, is a non-pathogenic symbiont of many invasive North American crayfish species (Tilmans et al. 2014); however, the introduction of *A. astaci* into Europe has resulted in large-scale mortalities in native crayfish populations, including: *Austropotamobius pallipes, Astacus astacus* and *Astacus leptodactylus*. In some cases, their local extinction is possible and has been noted in

Box I.

1) Co-transportation of pathogens

The invasive Asian eel (*Anguilla japonica*) brought with it the parasitic swim-bladder nematode *Anguillicoloides* crassus, which has caused high mortalities in native European eels (*Anguilla anguilla*) and significantly affected the sustainability of future European populations (Peeler et al. 2011). The OIE (World Organisation for Animal Health) – listed pathogen, *Bonamia ostreae* has caused decimation of native oysters (*Ostrea edulis*) in Europe, when it arrived with cultivated American populations of *O. edulis* for aquaculture in the late 1970s (Peeler et al. 2011).

The ornamental trade has been implicated in the introduction of the chytrid *Batrachochytrium dendrobatidis*; a pathogenic agent partly responsible for the global decline of amphibians and species extinctions (Fisher and Garner 2007). The trade of freshwater molluscs has long caused concern about the potential for snail-mediated zoonotic diseases as they can act as intermediate hosts for parasites of significance to humans and livestock (Ng et al. 2016), e.g., angiostrongyliasis in humans caused by the parasitic nematode *Angiostrongyliasis cantonensis* co-introduced with the invasive snails *Pomacea canaliculata* and *Pomacea maculata*.

Symbionts co-transported with INNS may be known pathogens which impact on wildlife in an expanded range (e.g., white spot syndrome virus; Mrugała et al. 2015), or their pathogenic potential may only be revealed when presented with new and susceptible hosts (e.g., the impact of *Aphanomyces astaci* on native white-clawed cray-fish in Europe; Tilmans et al. 2014).

2) Co-transportation of commensal organisms

The killer shrimp, *Dikerogammarus villosus*, invaded the UK in 2010, carrying the gregarine protists *Uradi-ophora longissima* and *Cephaloidophora mucronata* characterised from Polish freshwaters (Ovcharenko et al. 2009; Bojko et al. 2013). Gregarines are common commensal organisms of invertebrates that cover a wide symbiotic to parasitic spectrum (Rueckert et al. 2019) and undergo sexual reproduction in the animal gut, releasing spores into the environment that are consumed by other organisms. *Uradiophora longissima* and *C. mucronata* appear to be commensal organisms that have co-invaded with their host and do not exhibit any controlling effect upon the killer shrimp population (Bojko et al. 2013). Further molecular and histological studies will better identify commensal species by screening native and invasive populations of high-risk groups, such as the Amphipoda.

3) Invading symbiomes

Assessing the symbiome of an organism requires the use of multiple tools, including both visualisation (microscopy) and diagnostic (molecular detection) techniques. By understanding the symbiome, we can explore co-infection and approach the invasion from a pathobiome perspective (Bass et al. 2019). The symbiome of the demon shrimp *Dikerogammarus haemobaphes*, a European invader originating from the Ponto Caspian region, has been shown to include viruses, bacteria, protists (including microsporidia) and metazoans (Bojko et al. 2019; Bojko and Ovcharenko 2019), identifying risks coupled with the invasion process (Bojko et al. 2015; Allain et al. 2020; Subramaniam et al. 2020). For example, the microsporidian parasite, *Cucumispora ormata*, has been shown to reduce the activity of *D. haemobaphes* and increase its rate of mortality, initiating population control at invasion sites and lowering he direct impact of the host on local biodiversity and the environment. In tandem, this parasite is also capable of infecting native *Gammarus pulex*, constituting a wildlife risk (Bojko et al. 2019).

Metabarcoding and metagenomic techniques provide us with a capacity to easily pre-screen native species before they may become translocated. Metabarcoding of the UK invasive *Homarus americanus* cuticle revealed 170 associated bacterial taxa, suggesting that these microbial symbionts may have the capacity to invade with their host (Meres et al. 2012). Without technologies like these being used to advance invasion science, we remain in the dark about the complete symbiome and its associated risks.

many regions (Mrugula et al. 2014). Box 1 details examples of known co-transported pathogens and their effects, co-transported symbionts and how symbiome research can help to assess invasion risks.

INNS in aquatic systems

Aquatic ecosystems are considered more vulnerable to the effects of INNS introduction and spread than terrestrial ecosystems (Thomaz et al. 2015). Aquatic ecosystems are highly connected, and freshwater catchments link terrestrial, estuarine, and marine habitats longitudinally as water moves downstream, providing corridors along which organisms can move easily (Ormerod et al. 2011). Sites at high-risk of INNS introduction occur where vector activity associated with key introduction pathways is high, such as ports, marinas, and aquaculture sites (Keller et al. 2011; Tidbury et al. 2016). Many aquatic organisms have larval stages, which facilitate their dispersal across large distances (Wood et al. 2005). Detection of aquatic INNS often occurs after populations have already established, due to their patchy distribution and low abundance in the early stages of invasion, and difficulty in detecting and identifying early life stages using standard morphological techniques (Ponchon et al. 2013). However, new technologies, such as environmental DNA (eDNA) monitoring, offer increased opportunity for early detection and monitoring for both the host INNS and the associated pathogens (Robinson et al. 2018).

Routes of introduction of aquatic INNS

The CBD categorizes the pathways of introduction of an invasive species into three main categories; movement of commodities (releases, escapes, contaminants), via transport (stowaway), or by dispersal (corridor, unaided) (Hulme et al. 2008; Pergl et al. 2020). The human-mediated spread of INNS in marine systems is predominantly through global shipping networks via transfer in ballast water or hull fouling on vessels (Tidbury et al. 2016; Bailey et al. 2020). A review of *BioInvasions Records* showed that in the last 8 years the most common pathway of introduction has been via transport (stowaway), and the most important CBD pathway category was "ship/boat ballast water" (Stranga and Katsanevakis 2021).

Releases and escapes via the ornamental trade and aquaculture are the most important pathway for freshwater species (Nunes et al. 2015; Stranga and Katsanevakis 2021). The aquatic ornamental animal trade is worth \$25 billion per annum worldwide and represents a significant invasion pathway (Padilla and Williams 2004). INNS are also introduced through the illegal trade of ornamental aquatic animals. Laws regulating the aquatic pet trade are often poorly communicated and enforced, and in some cases can increase unwanted introductions of banned species (Patoka et al. 2018).

Aquaculture production has expanded rapidly in recent years and global demands are expected to increase to meet the needs of the growing human population (Stentiford et al. 2017). The movement of non-native animals between countries for aquaculture can spread INNS, and the open nature of many aquaculture sites to their surrounding environment can mean that INNS and their symbionts can be released into those environments (Atalah and Sanchez-Jerez 2020). The biggest risk to aquaculture production and growth has been identified as disease (Jennings et al. 2016), which highlights the importance of potential invasive aquatic pathogens and the need to control emerging disease threats. The increasing pressures on aquaculture to support global food security makes minimizing pathogen spill-over to the environment and wildlife, and vice versa, a critical priority to improve both the efficiency of production and ensure environmental sustainability (Stentiford et al. 2020). Bait used in recreational fishing is a potential pathway for pathogen introduction and dispersal if anglers dispose of bait or storage water/sediment into aquatic systems (Mahon et al. 2018). Discharge of effluent water from aquaria has also been identified as a high risk for incidental INNS release (Duggan 2010). Transport of live aquatic animals also means that the water in which they are transported becomes a potential source of non-native microbes (Amaral-Zettler et al. 2018). Furthermore, the transit of live animals can produce stressful conditions that can change the microbial communities that they harbour, often leading to disease (Smith et al. 2012). Analysis of imported fish and their carriage water through the supply chain showed increased levels of opportunistic pathogens such as *Vibrio* spp. (Amaral-Zettler et al. 2018).

Climate change can also facilitate natural range expansion of holobionts (Cottier-Cook et al. 2017). Increased water temperatures, altered hydrodynamics and more frequent extreme weather events are all predicted to increase the rate of aquatic species invasions (Rahel and Olden 2008). Rising water temperatures may mean that more ornamental species, and their symbionts are able to survive and establish. Warmer temperatures also allow pathogenic microbes to complete their life cycle more rapidly and attain higher population densities, increasing their virulence (Dutta and Dutta 2016).

In order to address the knowledge gap between INNS and their symbionts, we propose a literature-based workflow for compiling existing knowledge on a host's symbiome, members of which could be co-transported with INNS. This information is essential for assessing the consequences posed by co-transportation, or any INNS introduction to a new area. Such risks fall into three main categories: 1) pathogenic threats to native hosts or to species cultured or harvested for consumption or trade, 2) trade and legislative implications; for example, listed pathogens being introduced to regions previously considered free of them, and 3) effects of, or changes, to the invading species' symbiome in a new range, conferring novel ecological/behavioural characteristics on the invader.

Material and methods: Literature search methodology

A list of incoming aquatic INNS of concern to the UK was compiled from the lists of Roy et al. (2014) and a GBNNSS horizon scanning exercise (GBNNSS 2019). Seventy-seven aquatic INNS were identified from these lists (see Table 1). The literature searches were completed between August-October 2020.

To perform the literature search, both PubMed and Google Scholar were used to develop the best methodology (Figure 2). Figure 2 illustrates the workflow options and key considerations for choosing which database to search. Each has different characteristics that may preferentially suit different investigations. Both are subscription-free. The search terms used in this paper are given below; these can be adapted as required. This process can be used/adapted for non-aquatic species and with respect to any geographic region.

PubMed

(Species or genus name[#] [All Fields]) AND (microbiome[Title/Abstract] OR symbio*[Title/Abstract] OR pathogen*[Title/Abstract] OR parasit*[Title/Abstract] OR protist[Title/Abstract] OR protozoa[Title/Abstract] OR bacteria*[Title/Abstract] OR virus[Title/Abstract] OR host[Title/Abstract] OR reservoir[Title/Abstract] OR vector[Title/Abstract] OR infection [Title/Abstract])

Google Scholar

"Species name[#]" AND pathogen OR parasite OR commensal OR symbiont OR protist OR bacteria OR virus

[#]In cases where INNS taxa have recently been subject to taxonomic changes or are taxonomically ambiguous, multiple searches using alternative but equivalent names may be required.

Table 1. Non-native species at risk of arriving in the UK, as defined by Roy et al. (2014) and GB-NNSS (2019), and the results of literature searches as described in the main text. **[x]** is the number of publications informative about co-transported pathogens/symbionts, from which information was extracted and the publication cited in Suppl. material 1. When x < 4 in PubMed searches; informative publications were also searched for at INNS genus level (filtering to this degree was not possible or practical with Google Scholar). References for all of the informative publications selected are in Suppl. material 1 and Suppl. material 3. Taxonomic abbreviations: AL = Algae; AN = Annelida; ANG = Angiosperms; BR = Bryozoa; CH = Chordata (CH-U = Urochordata, CH-P = Pisces, CH-A = Amphibia, CH-R = Reptilia, CH-A = Aves, CH-M = Mammalia); CR = Crustacea; CT = Ctenophora; EC = Echinodermata; MO = Mollusca; NE = Nemertea; PL = Platyhelminthes; PO = Porifera.

| Species name | Common name | Taxon | PubMed Genus | PubMed | Google Scholar |
|---|---------------------------|-------|----------------|------------------|--------------------|
| - | | | search [X] | Species | Species search |
| | | | | search [X] | [X] |
| Aglaothamnion halliae | Brazilian red alga | AL | 1 [1] | 0 | 34 [0] |
| Antithamnion pectinatum | Australasian red alga | AL | 2 [0] | 0 | 40 [0] |
| Caulerpa taxifolia | killer alga | AL | 43 | 43 [8] | 2660 [4] |
| Gracilaria vermiculophylla | rough gar weed | AL | 90 | 6 [4] | 1140 [4] |
| Rugulopteryx okamurae | Asian fan weed | AL | 0 | 0 | 12 [0] |
| Eudistylia polymorpha/ Bispira polyomma | giant feather duster worm | AN | 1 [1] | 0 | 6 [0] |
| Marenzelleria wireni | red gilled worm | AN | 1 [1] | 0 | 17 [0] |
| Limnobium spongia | American frog's-bit | ANG | 68 | 0 | 128 [1] |
| Saururus cernuus | swamp lily | ANG | 58 [0] | 2 [0] | 474 [0] |
| Trapa natans | water chestnut | ANG | 17 [1] | 7 [1] | 1820 [0] |
| Zostera japonica | Japanese seagrass | ANG | 98 [71] | 1 [1] | 563 [4] |
| Schizoporella errata | branching bryozoan | BR | 0 | 0 | 209 [0] |
| Ommatotriton ophryticus | northern banded newt | CH-A | 0 | 0 | 21 [0] |
| Tadorna ferruginea | ruddy shelduck | CH-A | 21 | 10 [10] | 562 [15] |
| Threskiornis aethiopicus | African sacred ibis | CH-A | 7 [5] | 4 [2] | 435 [2] |
| Aonyx cinerea | short clawed otter | CH-M | 232 | 2 [2] | 166 [5] |
| Castor canadensis | American beaver | CH-M | 486 | 27 [25] | 3580 [12] |
| Myocaster coypus | coypu | CH-M | 52 | 51 [43] | 2270 [27] |
| Ondatra zibethicus | muskrat | CH-M | 58 | 42 [42] | 2650 [2 7] |
| Babka gymnotrachelus | racer goby | CH-P | 2 [2] | 2 [2] | 80 [8] |
| Carassius gibelio | Prussian carp | CH-P | 516 | 30 [20] | 1670 [30] |

| Species name | Common name | Taxon | PubMed Genus | PubMed | Google Scholar |
|---------------------------------|--------------------------------------|-------|----------------|------------------|--------------------|
| - | | | search [X] | Species | Species search |
| | | | | search [X] | [X] |
| Gambusia holbrooki | eastern mosquito fish | CH-P | 445 | 15 [5] | 2660 [7] |
| Micropterus salmoides | largemouth bass | CH-P | 1,939 | 131 [74] | 9200 [39] |
| Neogobius fluviatilis | monkey goby | CH-P | 44 | 6 [6] | 400 [15] |
| Neogobius melanostomus | round goby | CH-P | 44 | 35 [27] | 2050 [33] |
| Oncorhynchus gorbuscha | pink salmon | CH-P | 1,776 | 30 [25] | 3560 [32] |
| Proterorhinus marmoratus | Black Sea tubenose goby | CH-P | 9 [6] | 2 [2] | 383 [12] |
| Proterorhinus semilunaris | western tubenose goby | CH-P | 9 | 7 [4] | 209 [11] |
| Pterois volitans | red lionfish | CH-P | 13 [6] | 8 [3] | 1140 [12] |
| Umbra pygmaea | eastern mud minnow | CH-P | 5 [4] | 0 | 215 [1] |
| Chelydra serpentina | snapping turtle | CH-R | 22 | 21 [11] | 2180 [14] |
| Chrysemys picta | painted turtle | CH-R | 21 | 20 [12] | 2860 [14] |
| Ciona savignyi | sea squirt | CH-U | 123 | 123 [12] | 1120 [4] |
| Styela plicata | pleated tunicate | CH-U | 35 [6] | 15 [2] | 1350 [7] |
| Cercopagis pengoi | fishhook water flea | CR | 1 [0] | 1 [0] | 624 [0] |
| Chelicorophium robustum | A Ponto-Caspian amphipod | CR | 0 | 0 | 24 [0] |
| Chelicorophium sowinskyi | A Ponto-Caspian amphipod | CR | 0 | 0 | 13 [0] |
| Cherax destructor | common yabby | CR | 81 | 10 [6] | 1420 [7] |
| Dikerogammarus bispinosus | A Ponto-Caspian amphipod | CR | 22 [5] | 0 | 27 [0] |
| Dyspanopeus sayi | Say's mud crab | CR | 2 [1] | 2 [1] | 172 [0] |
| Echinogammarus ischnus | bald urchin shrimp | CR | 29 [21] | 0 | 322 [2] |
| Echinogammarus trichiatus | curly haired urchin shrimp | CR | 29 [21] | 3 [3] | 59 [3] |
| Echinogammarus warpachowskyi | A Ponto-Caspian amphipod | CR | 29 [21] | 0 | 16 [0] |
| Hemigrapsus sanguineus | Asian shore crab | CR | 24 | 6 [5] | 251 [4] |
| Hemigrapsus takanoi | brush-clawed shore crab | CR | 24 | 0 | 138 [3] |
| Homarus americanus | American lobster | CR | 119 | 63 [38] | 8230 [42] |
| Jaera istri | A Ponto-Caspian isopod | CR | 3 [2] | 1 [1] | 72 [1] |
| Limnomysis benedeni | A Ponto-Caspian mysid | CR | 1 [0] | 1 [0] | 169 [0] |
| Marsupenaeus japonicus | kuruma prawn | CR | 2,088 | 173 [65] | 4930[28] |
| Megabalanus coccopoma | titan acorn barnacle | CR | 4 [0] | 0 | 108 [0] |
| Megabalanus tintinnabulum | sea tulip | CR | 4 [0] | 0 | 130 [1] |
| Mytilicola orientalis | red oyster worm | CR | 15 [0] | 4 [0] | 349 [0] |
| Neocaridina davidi/ Neocaridina | cherry shrimp | CR | 8 [4] | 1 [0] | 93 [1] |
| heteropoda | | | | | |
| Obesogammarus crassus | A Ponto-Caspian amphipod | CR | 0 | 0 | 78 [2] |
| Obesogammarus obesus | A Ponto-Caspian amphipod | CR | 0 | 0 | 45 [1] |
| Orconectes rusticus | rusty crayfish | CR | 21 [15] | 3 [2] | 1280 [5] |
| Paramysis lacustris | A Ponto-Caspian mysid | CR | 0 | 0 | 88 [0] |
| Pontogammarus robustoides | A Ponto-Caspian amphipod | CR | 2 | 1 [1] | 250 [3] |
| Procambarus fallax | marbled crayfish | CR | 484 | 1 [1] | 323 [8] |
| Rhithropanopeus harrisii | Harris' mud crab | CR | 2 [1] | 1 [1] | 1220 [11] |
| Mnemiopsis leidyi | American comb jelly sea walnut? | СТ | 36 | 12 [6] | 2860 [15] |
| Asterias amurensis | Northern Pacific seastar | EC | 38 | 8 [4] | 1890 [6] |
| Bellamya chinensis | Chinese mystery snail | MO | 27 [17] | 1 [0] | 90 [3] |
| Corbicula fluminalis | Asian clam | МО | 37[18] | 0 | 222 [0] |
| Dreissena rostriformis bugensis | quagga mussel | МО | 79 | 10 [2] | 683 [2] |
| Geukensia demissa | Atlantic ribbed mussel | МО | 10 | 9 [4] | 1750 [4] |
| Lithoglyphus naticoides | gravel snail | МО | 4 [4] | 3 [3] | 373 [4] |
| Mulinia lateralis | dwarf surf clam | МО | 4 [3] | 3 [2] | 906 [2] |
| Ocinebrellus inornatus | Japanese sting winkle | МО | 0 | 0 | 146 [0] |
| Potamocorbula amurensis | Amur river clam | мо | 0 | 0 | 887 [0] |
| Rapana venosa | veined rapa whelk | мо | 12 | 7 [3] | 965 [4] |
| Sinanodonta woodiana | Chinese giant mussel | MO | 56 | 16 [3] | 671 [4] |
| Theora lubrica | Asian semele | мо | 0 | 0 | 162 [0] |
| Xenostrobus securis | pygmy mussel | мо | 1 [0] | 1 [0] | 177 [2] |
| Cephalothrix simula | A NW Pacific Ocean nemertean worm | NE | 2 [2] | 2 [2] | 89 [7] |
| Gyrodactylus salaris | salmon fluke | PL | 422 | 104 [0] | 2710 [0] |
| Celtodoryx ciocalyptoides | cauliflower sponge | РО | 1 [0] | 0 | 21 [0] |



Figure 2. Workflow for investigating existing data relating to symbionts (including pathogens) of current and potential INNS. The bullet points in each box indicate key considerations for each step of this customisable process. The list of factors in gray text influence whether PubMed or Google Scholar (or both) would be more appropriate for the particular species being researched.

Results

Using PubMed; 34 of the 77 aquatic INNS were found to have no relevant literature relating to any known symbiotic species or pathogens. At genus level this number falls to 23; however, the relevance of symbionts and potential pathogens associated with the genus-level compared to the target species is uncertain but aids prediction. Symbiont and pathogen information extracted from the literature search for each species is listed in Suppl. material 1.

There were nine taxa for which species-level symbiont/pathogen data were published in 20+ papers; *Neogobius melanostomus, Homarus americanus, Oncorhynchus* gorbuscha, Carassius gibelio, Micropterus salmoides, Castor canadensis, Marsupenaeus japonicus, Myocaster coypus, and Ondatra zibethicus. The importance of these species in aquaculture, fisheries and human health is likely to explain their dominance within the literature. Homarus americanus, Marsupenaeus japonicus and Oncorhynchus gorbuscha are all highly valuable aquaculture species. Carassius gibelio and Micropterus salmoides are associated with the ornamental trade and recreational angling respectively. Castor canadensis, Myocaster coypus and Ondatra zibethicus carry multiple pathogens of human importance (see Suppl. material 1). The results from PubMed and Google Scholar show some similarity. For taxa with little relevant literature, Google Scholar was more likely to return relevant data. As shown in Table 1, only 26 of the 77 aquatic INNS returned no relevant literature through Google Scholar in comparison to 34 from PubMed. For taxa with more literature; PubMed returned a larger proportion of useful papers in fewer results, and although these were usually also identified in Google Scholar, significantly more manual sifting of results in order to find these papers was required. For example, *Marsupenaeus japonicus* had 65 relevant papers selected from PubMed, but only 28 were identified from the first 100 Google scholar results despite a vastly larger overall return. This is likely to be because PubMed allowed for a more targeted search. We found using both PubMed and Google Scholar in parallel gives the most comprehensive picture.

Discussion

PubMed search tools enabled a more accurate search as highly structured search criteria could be applied to just the title and abstract of papers, allowing a more focused search. However, the library of literature available in PubMed is smaller than on Google Scholar, and data from some figures and tables is not screened, sometimes leading to the omission of useful information. Google Scholar returned a significantly higher number of publications; the library of literature is much larger and it also scans grey literature and academic thesis repositories. However, Google Scholar also returns a much higher rate of irrelevant results which require significant manual sifting, in part because it scans the references of articles, and because the search cannot be narrowed by abstract. It is also important to scrutinize the source of literature from Google Scholar as it includes non-peer reviewed literature which may not always be suitable depending on the remit of the literature search. Haddaway et al. (2015) provides evidence to show that Google Scholar can be a powerful resource when used alongside other search methods; but is best used as a complementary tool.

Where there is a knowledge gap regarding the symbionts and pathogens of the target species, expert advice may be highly beneficial. This is likely to be the case for many known and potential INNS in most countries. Collaborative expert-elicitation is also a highly valuable tool within the field of biological invasion policy and has been implemented in numerous successful studies (Booy et al. 2017; Hughes et al. 2020; Peyton et al. 2019; Roy et al. 2014, 2017, 2018). These methods have been refined to ten guiding principles to consider within expert-elicitation to increase the effectiveness of this tool (see Roy et al. 2020).

When assessing the reliability of reports of co-transported pathogens in the literature, it is important to consider the methods used for their identification. Genetic signatures of pathogens may be associated with particular host samples in the literature, but these do not necessarily represent infections of those hosts; for example they could be passing through the gut and/or infecting host food items. Visualization techniques such as histopathology or in situ hybridization can be used to more precisely determine host-pathogen relationships initially inferred from molecular-only data. The use of such complementary techniques is recommended for research seeking to fill knowledge gaps such as those identified in this paper.

Current INNS legislation

Recognition of the negative impacts of INNS is evidenced by the increase in legislation and policy that aims to mitigate or reduce INNS impacts. Aichi Target 9 of the CBD commits signatories, of all member parties, to minimize new introductions of INNS, and control and eradicate priority species (UNEP 2011). This commitment is reflected in European legislation, including Regulation (EU) No 1143/2014 on the prevention and management of the introduction and spread of invasive species (EU 2014). There are additional legislative drivers within the EU to reduce the introduction and spread of INNS as a driver of environmental degradation (Water Framework Directive 2000/60/EC) and as an indication of human pressures (Marine Strategy Framework Directive 2008/56/EC).

Current Pathogen legislation

The World Organisation for Animal Health (OIE) has the mandate to prevent the spread of important animal pathogens, including those of aquatic animals (defined as amphibians, crustaceans, fish, and molluscs). OIE standards are recognized by the World Trade Organisation and applied within its Sanitary and Phytosanitary (SPS) agreement. The 182 members of the OIE include all major economies. National and supra-national (e.g., EU laws) need to be consistent with OIE standards. The EU Regulation 2016/429 (Animal Health Law) provides the legal basis to prevent the spread of important listed infectious pathogens. The criteria necessary for listing a pathogen include a significant negative impact on farmed animal production or biodiversity (through biosecurity, contingency planning, surveillance, and eradication) and will be applicable from 21 April 2021 (Council of Europe 2019).

Pathogens are recognized in the International Council for the Exploration of the Sea (ICES) Code of Practice on the "Introductions and Transfers of Marine Organisms", which has existed in some form since 1973 (ICES 2005). This sets out recommended procedures for the introduction of INNS for commercial reasons (e.g., aquaculture, bait) to ensure they are free of known pathogens (Turner 1988).

Future INNS policy recommendations

The CBD places a focus on the prevention of INNS introductions (followed by early detection and rapid response). Risk assessments of INNS are identified as a key element of the risk analysis process which is required for prioritising INNS for management. At an international level, countries under the SPS agreement must provide a risk assessment to support measures to prevent disease spread that go beyond international

(OIE) standards. Co-transported pathogens, however, cannot be risk assessed, or regulated and controlled if they are unknown and unquantified. Therefore, we recommend 1) more intensive study of INNS and their associated symbionts (including known and potential pathogens), using both experimental and diagnostic evidence to support evidencing INNS risk assessments; 2) identification of high-risk potential INNS and recent invaders and targeted investigation; and 3) investigation of which INNS taxa might co-transport high risk pathogens, based on what we know of the pathogens/ symbionts of those groups more generally.

As suggested by Roy et al. (2017), the inclusion of information on pathogens within alien species databases, including the communication of such information, is critical to the success of management programs that aim to mitigate the impacts of pathogens co-transported with INNS. Future priorities should be to collect baseline information on the distribution and population dynamics of parasites, hosts and vectors, to determine the relative importance of invasion pathways, and to develop methods for predicting host shifts, parasites-host dynamics and the evolution of alien pathogens (Roy 2016). Many aspects of the study and management of emerging infectious diseases and biological invasions work in parallel. Collaboration across disciplines is important to effectively tackle these issues, such as adopting the One Health framework (Ogden et al. 2019; Bojko et al. 2020).

Conclusion

Invasion biology needs more robust methods for reliably evaluating the risks associated with INNS introductions (Kumschick et al. 2015). One of the most important factors to consider as part of risk assessments is evaluating the symbiome of INNS. Therefore, there is a need to better understand symbionts associated with INNS in order to evaluate the potential threat of emerging co-invasive pathogens as part of the INNS risk assessment processes (including horizon scanning). The workflow proposed in this paper uses a tested set of search terms in both PubMed and Google Scholar to thoroughly scan any available literature. This workflow aims to allow comprehensive data gathering of pathogens potentially co-transported with INNS, and constitutes a simple yet powerful methodology for the robust and standardized assessment of symbionts associated with INNS. As such, it provides a crucial step towards addressing the knowledge gaps regarding co-transportation of symbionts, facilitating integration of such knowledge into INNS risk assessment.

While limitations exist with respect to INNS data, the increasing use of histological, eDNA, and molecular diagnostics also offer new opportunities for monitoring INNS, potentially enabling the capture of pathological data more easily. Innovative modelling approaches, such as those using evolutionary trait-based frameworks (Barwell et al. 2020), can also inform horizon scanning and risk assessment to identify potentially impactful pathogens.

The introduction of INNS is widely recognized as important in both introducing known pathogens and a driver for the emergence of new pathogens (Peeler et al. 2011). There is a need at both international and national level for a collaborative approach to the assessment of INNS, efficient resource use and the formulation of guidance and risk assessment tools to both prevent and control the introduction of INNS and their symbionts. INNS do not recognize political boundaries so their effective management, particularly within the marine environment, requires transboundary coordination and collaboration.

Improved awareness raising, in particular across key sectors and stakeholder groups, will be important for managing the threat of INNS and their symbionts. The proposed amendments to risk assessment processes should aid in the more appropriate identification of INNS risk, but this will also need to be incorporated into other aspects of risk analysis including horizon scanning, risk management and prioritization. Further, robust and standardized prevention and mitigation approaches are needed globally to implement suitable actions once a species has been prioritized. For example, pathway management, border checks (to include molecular based screening for symbionts) and quarantine for intentionally introduced INNS, and routine monitoring and rapid response following detection of unintentionally introduced INNS. The use of molecular based tool sets is increasingly becoming a go to option for the detection of INNS and will be a necessity for the detection symbionts they may carry. Explicit consideration of symbionts and potential for disease emergence should also be made within assessments undertaken prior to the translocation of both INNS and native species for conservation or assisted colonization purposes such as for aquaculture.

This issue is now more pressing than ever: climate change could act synergistically with other stressors, to increase the impacts of invading pathogens. Rising water temperatures may mean more INNS and their pathogens are able to survive and establish in the UK. Furthermore, the increasing global demands on aquaculture production, mean that impacts arising from emerging aquatic diseases are increasing in frequency, and have increasingly diverse and serious economic implications.

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Appendix

Table A1. Glossary.

| Symbiont | Host-associated organisms and viruses, including long-term or transitory associations, epibionts | | | |
|---------------|--|--|--|--|
| | and endobionts. | | | |
| Pathogen | A symbiont that causes disease in certain hosts under certain conditions. Its presence need not | | | |
| | result in disease. Often used interchangeably with 'parasite'. | | | |
| Parasite | A symbiont that derives nutrition/material resource from its host in one of several ways, not | | | |
| | necessarily resulting in disease. Includes indirect feeding types including host stomach contents or | | | |
| | metabolic products. Often used interchangeably with 'pathogen'. | | | |
| Enemy Release | INNS can lose their parasites as they move into a new range, thus increasing host biological fitness | | | |
| Hypothesis | as the resources used to fight the infection are no longer required (Keane and Crawley 2002). | | | |
| Parasite | INNS can acquire parasites from the new range, resulting in parasite spillback to native species | | | |
| Spillback | by increasing the population of infected individuals (Sheath et al. 2015), thus changing disease | | | |
| | dynamics of infected native species at individual and population scale levels (Kelly et al. 2009). | | | |
| Parasite | When parasites from INNS are transmitted to susceptible native host species (Power and Mitchell | | | |
| Spillover | 2004). | | | |
| Disease | INNS may act as 'disease facilitators' by aiding the physical transfer of parasites through acting | | | |
| Facilitation | as vectors or a reservoir, or via their role in habitat alteration which may improve parasite | | | |
| Hypothesis | environmental conditions (Chalkowski et al. 2018). | | | |
| Co-transport | Organisms which are transported with an alien host to a new location outside of their native range | | | |
| | (Lymbery et al. 2014) | | | |
| Heteroecious | A parasite that requires at least two hosts. | | | |
| parasites | | | | |

Supplementary material I

Table S1

Authors: Rachel Foster

Data type: literature workflow results

- Explanation note: This table shows known pathogens, potential pathogens, and symbionts of each INNS found using the proposed literature search workflow, with specific references (superscript numbers) listed below the table.
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Link: https://doi.org/10.3897/neobiota.69.71358.suppl1

Supplementary material 2

Table S2

Authors: Rachel Foster

Data type: rerefence list

- Explanation note: This table lists all the references found using the proposed workflow as shown in Table 1 in the manuscript for PubMed.
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Link: https://doi.org/10.3897/neobiota.69.71358.suppl2

Supplementary material 3

Table S3

Authors: Rachel Foster

Data type: Reference list

- Explanation note: This table lists all the references found using the proposed workflow as shown in Table 1 in the manuscript for Google Scholar.
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Growing up in a new world: trait divergence between rural, urban, and invasive populations of an amphibian urban invader

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Abstract

Cities are focal points of introduction for invasive species. Urban evolution might facilitate the success of invasive species in recipient urban habitats. Here we test this hypothesis by rearing tadpoles of a successful amphibian urban coloniser and invader in a common garden environment. We compared growth rate, morphological traits, swimming performance, and developmental rate of guttural toad tadpoles (*Sclerophrys gutturalis*) from native rural, native urban, and non-native urban habitats. By measuring these traits across ontogeny, we were also able to compare divergence across different origins as the tadpoles develop. The tadpoles of non-native urban origin showed significantly slower developmental rate (e.g., the proportion of tadpoles reaching Gosner stage 31 or higher was lower at age 40 days) than tadpoles of native urban origin. Yet, tadpoles did not differ in growth rate or any morphological or performance trait examined, and none of these traits showed divergent ontogenetic changes between tadpoles of different origin. These findings suggest that prior adaptation to urban habitats in larval traits likely does not play an important role in facilitating the invasion success of guttural toads into other urban habitats. Instead, we suggest that evolutionary changes in larval traits after colonization (e.g., developmental rate), together with decoupling of other traits and phenotypic plasticity might explain how this species succeeded in colonising extra-limital urban habitats.

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Keywords

AIAI hypothesis, development, growth rate, invasion biology, morphology, performance, tadpole, urban evolution

Introduction

Invasive species pose a major threat to global biodiversity, human wellbeing, and the economy (Pejchar and Mooney 2009; Gallardo et al. 2016; Mollot et al. 2017; Hanley and Roberts 2019; Diagne et al. 2021). The introduction of invasive populations is fundamentally linked to human activities such as global transport and habitat alteration (Pyšek et al. 2010; Blackburn et al. 2011; Hill et al. 2017b; McLean et al. 2017). Heavily-modified landscapes, like cities, harbour a significant proportion of invasive species (Cadotte et al. 2017; Gaertner et al. 2017), moreover, urban land cover is predicted to expand rapidly with rising human populations - especially in countries within biodiversity hotspots (Seto et al. 2012). Urban areas are focal points of introduction for alien biota due to global trade and transportation (Padayachee et al. 2017; Bullock et al. 2018; Gippet et al. 2019; Rivkin et al. 2019), and they show distinct habitat characteristics that make cities more similar to each other than when comparing each city to its surrounding less-disturbed environment (McKinney 2006; Grimm et al. 2008). Those habitat characteristics (e.g., reduced native biodiversity and predator species richness, higher rates of impervious surface area, and the urban heat-island effect; McKinney 2006; Grimm et al. 2008; Ramamurthy and Bou-Zeid 2017) can also facilitate, if not promote, the establishment of invasive populations (McKinney 2008; Santangelo et al. 2018; Des Roches et al. 2020; Reed et al. 2020). Thus, if an alien population has established in one city, there may be an increased likelihood that propagules from that population could disperse to other cities where they encounter similar environmental conditions due to the homogenisation of urban landscapes, making their establishment and spread more likely (McKinney 2006; Rivkin et al. 2019; Reed et al. 2020).

Recently, evolutionary biologists have begun studying the adaptive divergence of traits in urban populations compared to populations from rural habitats (Johnson and Munshi-South 2017; Santangelo et al. 2018). Research has shown that urban populations can evolve to cope with novel challenges that cities pose (e.g., Brans et al. 2017a, b; Tüzün et al. 2017; Putman et al. 2019; Corsini et al. 2021). However, whether and how adaptation to urban habitats facilitates the establishment success of populations transported to cities outside their natural ranges remains largely unknown (Reed et al. 2020; Borden and Flory 2021). The "Anthropogenically Induced Adaptation to Invade" (AIAI) hypothesis (Hufbauer et al. 2012) postulates that anthropogenically modified habitats force adaptations in native populations to the anthropogenic selection regime, and given that altered landscapes are well connected due to global trade, the likelihood of prior adapted populations being transported to another anthropogenically modified habitat is high (e.g., Brady and Hay 2020). As human landscape

alterations lead to homogenisation of habitats (McKinney 2006), a prior adapted population will display a phenotype that is less likely to be mismatched in the recipient altered habitat compared to when being introduced to an unaltered habitat (Hufbauer et al. 2012). Thus, cities – as highly modified and interconnected habitats - pose an excellent opportunity to test the AIAI hypothesis.

To date, few studies have investigated whether prior adaptation to urban habitats facilitates invasion success in introduced habitats (Borden and Flory 2021). One such prior adaptation shown to provide invasive populations an advantage in urban landscapes are adaptive shifts in thermotolerance which occur in human-modified habitats before colonisation of novel ranges of the little fire ant (Wasmannia auropunctata; Foucaud et al. 2013) and several species of parrot (Jackson et al. 2015; Strubbe et al. 2015). Furthermore, several studies have shown that prior adaptation to the selective regime of the introduced range enhances invader fitness (Rey et al. 2012; Vahsen et al. 2018; Saarinen et al. 2019; Sherpa et al. 2019; Alzate et al. 2020). To study if and how urban evolution leads to prior adaptation in invasive populations some key attributes are required of the model system. First, there needs to be a population in an urban and in a rural habitat (preferably in close proximity) in the native range where the urban population is confronted with novel environmental challenges compared to the ancestral rural environment (Hufbauer et al. 2012; Borden and Flory 2021). Second, an invasive population must have been established in an urban area and this population must have originated from the native urban population (Hufbauer et al. 2012; Borden and Flory 2021). Third, to study evolutionary changes, the traits examined must be shown to be genetically fixed (e.g., by using a common garden experiment; Hufbauer et al. 2012; Lambert et al. 2020; Borden and Flory 2021).

Amphibians provide an excellent model system for examining the relationship between urban adaptations and invasions. Currently, there are more than 120 amphibian species with recognised invasive populations globally (Measey et al. 2020a), many of which were established through urban/suburban landscapes (e.g., Rebelo et al. 2010; Moore et al. 2015; Tingley et al. 2015) or other anthropogenically-altered habitats (e.g., aquacultural areas; Wang et al. 2019). Furthermore, a number of traits (e.g., growth rate, body shape, and developmental rate) have been demonstrated to diverge between rural/urban, and native/invasive amphibian populations (Iglesias-Carrasco et al. 2017; Iglesias-Carrasco et al. 2018; Eakin et al. 2019) and amphibians are well-known to show rapid evolution particularly in larval traits (e.g., Skelly and Freidenburg 2008; Nunes et al. 2014; Melotto et al. 2020). For example, comparisons of rural and urban populations have shown that wood frog tadpoles (Lithobates sylvaticus) in suburban pools were larger and developed earlier compared to tadpoles from rural pools (Eakin et al. 2019), also urban adult males tended to be larger and have a better body condition in several amphibian species compared to rural adult males (Iglesias-Carrasco et al. 2017). Thus, urban amphibian populations, especially of urban exploitative species, benefit from altered biotic and abiotic factors in urban wetlands, such as reduced interspecific competition and longer hydroperiods (i.e., wetland permanency) (Rubbo and Kiesecker 2005; Hassall 2014; Hill et al. 2017a). This trend is continued for invasive

amphibian populations that benefit from reduced competition by growing and developing faster, presumably to begin reproducing sooner (Phillips 2009; Pujol-Buxó et al. 2020). There has not been much work done on how tadpole swimming performance and the underlying morphological traits (i.e., body shape and tail morphology; Van Buskirk et al. 1997; Dayton et al. 2005; Teplitsky et al. 2005; Arendt 2010) diverge between rural and urban or between native and invasive populations. These traits, however, are known to change in response to novel selection regimes such as reduced predation and/or competition (i.e., larger bodies and smaller tail fins; Smith and Van Buskirk 1995; Relyea 2002; Relyea 2004; Dayton et al. 2005). Since urban bodies of water often represent habitats with reduced predation and interspecific competition for aquatic organisms (Rubbo and Kiesecker 2005; Hassall 2014), selection on morphological and performance traits may differ between rural and urban populations.

Here, we will examine the trait divergence in tadpoles of the guttural toad (Sclerophrys gutturalis) of three different origins in South Africa: native rural (Durban Rural), native urban (Durban Urban), and non-native urban (Cape Town, an invasive population that originated from Durban; Telford et al. 2019), within a common garden experiment. We focus on a suite of traits across development including tadpole body shape, tail morphology, and swimming performance. Furthermore, we examine whether these traits diverge across larval ontogeny between tadpoles of different origin. In doing so, we aim to test three hypotheses relating to the tadpoles' development, morphology, and performance capacity. Firstly, we predict that native rural tadpoles grow and develop slower than native urban tadpoles and urban invasive tadpoles grow and develop the fastest (i.e., relating to evolved increases in growth and developmental rate in urban/invasive populations; Sargent and Lodge 2014; Brans and De Meester 2018). Secondly, we expect that native rural tadpoles will have more slender bodies, but larger tail fins relative to body size, compared to native urban tadpoles and that invasive urban tadpoles have the bulkiest bodies with the smallest tail fins relative to body size (i.e., relating to reduced predation and competition in urban/invasive habitats; Rubbo and Kiesecker 2005; Hassall 2014; Hill et al. 2017a and following the findings of previous work on tadpole morphology; Smith and Van Buskirk 1995; Relyea 2002; Relyea 2004; Dayton et al. 2005). With these differences in growth, development and shape established, we then predict that native rural tadpoles will exhibit the fastest swimming speeds with native urban tadpoles being intermediary and invasive urban tadpoles to exhibit the slowest swimming speeds, owing to the expected differences in morphology and following the known effect of body and tail shape on performance (Van Buskirk et al. 1997; Dayton et al. 2005; Teplitsky et al. 2005; Arendt 2010).

Materials and methods

Study species

The guttural toad is a large bufonid (maximum snout-vent length (SVL)) of 140mm; du Preez et al. 2004), which is sexually dimorphic in body size (Baxter-Gilbert et al.

2020; Suppl. material 1: Fig. S1) and has a wide distribution in sub-Saharan Africa (Fig. 1A, B; du Preez et al. 2004; Telford et al. 2019). This habitat generalist can be found in a variety of environments including forests, grasslands, agricultural, and urban areas (du Preez et al. 2004; Channing et al. 2012; Baxter-Gilbert et al. 2020). Guttural toads breed in natural water bodies, such as shallow pools in rivers, and anthropogenic bodies of water, such as garden ponds and ditches. A single clutch can contain up to 25,000 eggs, laid in gelatinous strings (du Preez et al. 2004). Tadpoles usually develop over the course of five to six weeks with toadlets leaving the water as soon as the front legs have fully developed (du Preez et al. 2004; Suppl. material 1: Fig. S2).

The species has successfully established invasive populations in Mauritius, Réunion, and near Cape Town (Constantia, South Africa) (Measey et al. 2017; Telford et al. 2019; Measey et al. 2020b). Recent genetic analyses have confirmed that all three invasive populations originated from a clade located in the area around the port city of Durban in eastern South Africa (Telford et al. 2019). In the case of the invasive Cape Town population, guttural toads were most likely introduced as eggs or tadpoles within a shipment of aquatic plants at the end of the 1990s originating from a residential area of Durban (De Villiers 2006; Measey et al. 2017).

Sampling sites, animal collection, husbandry, and breeding

Breeding-sized adults (Suppl. material 1: Fig. S1) were collected from the end of November 2019 to the beginning of February 2020. We collected toads from two sites in the rural area surrounding Durban (hereafter referred to as "Durban Rural": 29°51'31"S, 30°43'18"E and 29°28'17"S, 31°13'25"E with 302 m and 54 m elevation above sea-level [a. s. l.], respectively), from two urban sites in and near Durban (hereafter referred to as "Durban Urban": 29°50'55"S, 31°00'30"E and 29°49'08"S, 30°56'37"E with 24 m and 194 m elevation a. s. l., respectively). Toads from the extra-limital range in Constantia near Cape Town (hereafter referred to as "Cape Town": 34°01'29"S, 18°26'03"E with 50-100 m elevation a. s. l.) were collected as part of an invasive species control operation (Davies et al. 2020a, 2020b). We chose the Durban Urban sites because they show similar habitat characteristics as the Cape Town sites, like high relative proportion of impervious surface area, well-established human populations, high numbers of buildings and roads, and the bodies of water within those urban sampling sites were either anthropogenically altered or created (e.g., fish ponds, fountains, or ditches) with heavily modified shorelines (Fig. 1D, E) and frequently contained ornamental fish species. All these characteristics are typical of urban environments (Rubbo and Kiesecker 2005; du Toit and Cilliers 2011; Moll et al. 2019) and have frequently been shown to drive adaptation in urban populations (e.g., Winchell et al. 2016; Corsini et al. 2021). Conversely, the Durban Rural sites have very low relative proportions of impervious surface area, few human settlements nearby with low human population densities and little human alteration. The bodies of water within our rural sites (i.e., a large lake and a series of ponds) are characterised by only very few anthropogenic modifications of the shoreline with riparian



Figure 1. Overview of the study system **A** a guttural toad (*Sclerophrys gutturalis*): this female was photographed in Cape Town **B** the species' natural and non-native distribution in South Africa. The approximate locations of sampling sites are demarcated for Durban Rural (green), Durban Urban (yellow), and Cape Town (red). Further we show the general appearance of **C** the Durban Rural **D** durban Urban, and **E** Cape Town sampling sites.

grasslands consisting of mainly native plants and the presence of native fish species (Fig. 1C).

Shortly after collection, toads were transported to an experimental facility located at the University of KwaZulu-Natal (Westville Campus) situated at one of our sampling locations for Durban Urban. In the facility, toads were housed by sex and collection site in large plastic mesocosms (110 cm L × 130 cm W × 50 cm H) until they were used for breeding. Each mesocosm contained at least two water bowls (~ 15 cm L × 10 cm W × 5 cm H) on a 10 cm layer of soil mixed with leaf litter collected outside of the greenhouse. Crickets (*Acheta domesticus*) were fed to adults *ad libitum* every other day.

To initiate breeding, we injected adults with a synthetic gonadotrophin, leuroprorelin acetate (Lucrin Depot, Abbott), diluted 1:20 with Ringer's solution using 0.666 ml of that dilution for females and 0.333 ml for males (Hamilton et al. 2005; Hudson et al. 2015). Breeding was induced from December 2019 to the beginning of March 2020 in batches of breeding pairs due to logistical reasons. This means that clutches were not all laid at the same time but rather breeding was carried out over the whole period described. However, we spread breeding for the different toad origins over this entire period. Injections took place at ~17:00 h. After a breeding pair was injected, pairs of male and female toads were placed into plastic containers (26×41 cm) filled to a height of 4 cm with aged tap water and left overnight. Usually, males would initiate amplexus shortly after being introduced to the female. The next morning at ~09:00 h containers were checked for fertilised clutches and adults were removed from the containers. All adults were used for
breeding only once. New clutches were cleaned from any faecal material and water was replaced with aged tap water and filled to a height of 15 cm in the plastic containers. We replaced water regularly with aged tap water and made sure tanks were well aerated and clean. Embryonal development was rapid and, after one day, most embryos had hatched. For our mesocosm experiment, this was considered as "age 0" day for tadpoles.

Mesocosm experiment

Prior to the experiment, large plastic mesocosms (110 cm L \times 130 cm W \times 50 cm H; n = 33) located in the experimental facility were filled with 600 L of tap water and left to age for a week. Subsequently, the water was inoculated with water from a standing water tank to induce establishment of phyto- and zooplankton communities within the mesocosms. This water tank was located in the greenhouse (i.e., preventing access from toads and fish) and had live aquatic vegetation and algae growing within it. After another week, 50 g of rabbit chow (Rabbit Pellets, Westerman's Premium; 9% protein, 1.25% fat, 0.75% calcium by weight) was added for additional nutrients (Semlitsch and Boone 2009). Mesocosms were placed under work benches so that half of the water surface was shaded and the other half received sunlight through the roof panels of the facility. After approximately one month, all mesocosms contained a visible phyto- and zooplanktonic community and were subsequently deemed ready for use. At the clutch age of three days, 1000 tadpoles from a single clutch (i.e., full siblings) were enumerated and introduced to a readied mesocosm where they would be housed until the end of the experiment. For a summary of the average daily maximum and minimum temperatures inside the mesocosms over the course of the experiment see Suppl. material 1: Fig. S3.

Sample sizes and measurements across ontogeny

At clutch age of three days, 20 tadpoles from one clutch were randomly selected for measurements (see below) and were returned to their respective mesocosms afterwards. At the age of ten days, and subsequently every ten days (i.e., age 20, 30, 40, 50 and 60 days), 20 tadpoles were randomly selected from each mesocosm, and measurement procedures were repeated (for sample sizes see Table 1). Note that for some clutches we were not able to conduct measurements on the scheduled day and instead conducted measurements on the next possible day (Suppl. material 1: Table S1). For all trait measurements, we made sure to only include tadpoles that did not show development past Gosner stage 41 (i.e., hindlimbs fully developed but tail not resorbed and front limbs did not emerge; Gosner 1960) as metamorphosis strongly affects the expression of the traits we examined in subsequent stages (Watanabe and Sasaki 1974; Gilbert 2000; Vitt and Caldwell 2013). Notes were made on any individuals that reached metamorphosis as they climbed on to floating platforms placed inside the mesocosms.

After the measurements (see below), tadpoles were returned to their respective mesocosms. We acknowledge that our replicates cannot be assumed to be fully independent **Table 1.** Sample sizes for guttural toad (*Sclerophrys gutturalis*) tadpoles across age: numbers below the specific traits correspond to the total number of tadpoles measured at the specific age. The total number of clutches used in this experiment was 10 (3/7), 14 (8/6), and 9 for Durban Rural, Durban Urban and Cape Town, respectively. The numbers in brackets correspond with the specific number of clutches derived from adults collected in either the first or second sampling location for Durban Rural or Durban Urban, respectively (see Methods). For a more detailed report of the sample sizes in this experiment see Suppl. material 1: Table S1.

| Traits | | Durban Ru | al | | Durban Urba | an | | Cape Town | |
|------------|------------------------------------|---|---|------------------------------------|---|---|------------------------------------|---|---|
| Age (Days) | SVL, body width, tail length | Body height, tail fin height, tail muscle height | Maximum velocity, maximum acceleration | SVL, body width, tail length | Body height, tail fin height, tail muscle height | Maximum velocity, maximum acceleration | SVL, body width, tail length | Body height, tail fin height, tail muscle height | Maximum velocity, maximum acceleration |
| 2-4 | 199 | 199 | 199 | 272 | 271 | 272 | 180 | 180 | 179 |
| 10-12 | 200 | 199 | 200 | 240 | 235 | 240 | 160 | 160 | 160 |
| 20 | 200 | 200 | 200 | 180 | 179 | 180 | 160 | 160 | 160 |
| 30 | 200 | 199 | 200 | 180 | 180 | 180 | 80 | 80 | 80 |
| 40-41 | 160 | 160 | 160 | 180 | 180 | 180 | 80 | 80 | 80 |
| 50 | 60 | 60 | 60 | 180 | 180 | 180 | 60 | 60 | 60 |
| 60 | 60 | 60 | 60 | 100 | 100 | 100 | 40 | 40 | 40 |

(e.g., a tadpole that was randomly picked for measurements at an age of three days might have been picked for another subsequent measurement at a higher age). However, since each mesocosm housed 1000 tadpoles at the start of the experiment, it is unlikely that a single tadpole was recurrently picked for measurements. As such our statistical analyses use the assumption that repeated measurements of an individual tadpole did not occur.

Morphological measurements and developmental rate

We measured standard morphological variables of tadpoles: SVL, tail length, body width, body height, tail muscle height, and tail fin height to 0.001 mm (Altig 2007; Suppl. material 1: Fig. S4). Images were scaled using millimeter paper in the background of tadpole pictures. Tadpole morphology was measured using the measurement function of the image and video analysis software Tracker (Open Source Physics, USA) (Brown and Cox 2009). To observe differences in body shape, we also calculated body volume (mm³) using the formula $\frac{3}{4} \times \pi x$.*SVI*. × hady width. × height $\frac{3}{4} \times \pi x$.*SVI*. × hady width. × height (e.g., tadpoles with bulkier bodies have higher values than tadpoles with more slender bodies). At the age of 40/41 days (see Table 1 for sample sizes, Suppl. material 1: Table S1), we also carefully examined tadpole images to see whether tadpoles had developed to, or past, Gosner stage 31 (i.e., well-developed hindlimbs; Gosner 1960) to study if developmental rate diverges between tadpoles of different origin. The purpose of using Gosner stage 31 as a threshold was to have a clear morphological marker (i.e., hindlimb; Gosner 1960) that is unambiguously identifiable from images of the tadpoles.

Performance measurements and video analysis

All tadpoles that underwent morphological measurements were also tested for swimming performance on the same day. Performance trials were carried out in a clear plexiglass tank (30×5 cm) filled with 3 cm of aged tap water (Wagener et al. 2021). A mirror was attached to the tank at an angle of 45° to enable us to record the movement of tadpoles using a dorsal and lateral point of view. At the start of the trial, an individual tadpole was gently placed inside the arena and left to acclimate for ~5 s. Water temperature was recorded using a standard digital thermometer. Tadpoles were tapped at the tail fin with a fine metal wire to initiate burst escape swimming for ~30 s. Videos of tadpoles swimming were recorded using a camera (Canon PowerShot G16) at 120 frames per second.

High-speed videos were recorded from a dorsal and lateral perspective to the tadpole, using an angled mirror attached to the tank. As a swimming bout we defined the movement of a tadpole, initiated by rapid lashes of the tail fin in response to an approach or a touch by the metal wire, from the beginning to the end of the displacement. Only swimming bouts that were carried out on a horizontal plane with a displacement at least 2 cm away from its initial position were selected. For each tadpole, we analysed three swimming bouts that were judged to yield the highest values for velocity and acceleration. Using the image and video analysis software Tracker (Brown and Cox 2009), we extracted the x- and y-coordinates from the videos and the displacement (mm) of the tadpole per frame was calculated. Videos were scaled using millimeter paper in the background of videos. We filtered the displacement data using a fourth order zero-phase shift low-pass Butterworth filter (Christodoulakis et al. 2010) and used a 12 Hz cut-off frequency which was determined as a 10th of the video recording frame rate. The Butterworth fourth order zero-phase shift low-pass filter is used to reduce noise in a data set by a given cut-off frequency (i.e., reducing the number of data points to filter noise in a data set) with the possibility to differentiate the filtered data into velocity (first derivate against time) and acceleration (second derivate against time) (Winter 2004; Erer 2007). From the filtered data we extracted maximum swimming velocity (mm \times s⁻¹) and maximum swimming acceleration (mm \times s⁻²) for each tadpole.

Statistical analysis

All statistical analyses were conducted using R version 4.0.1 (R Core Team 2020). Before analysis, we explored our data following Zuur et al. (2010). We did not find any unexplainable outliers and ensured the models did not contain strongly confounded predictor variables. All figures presented in the results section were created using the R package "ggplot2" (Hadley 2016). All morphological and performance traits were log-transformed prior to analysis. Following model analysis (see details below), we verified assumptions of normality and homoscedasticity of outliers prior to interpretation. Also, we conducted post-hoc multiple comparison tests between all origins (Durban Rural, Durban Urban, Cape Town) using the function "emmeans" from the R package "emmeans" (Lenth et al. 2018). All *p*-values generated for these comparisons were Tukey-adjusted (Lenth et al. 2018).

Ontogenetic differentiation of morphological and performance traits

To examine differences in growth rate and body volume, we fitted linear mixed effect models (LMM) using the R package "lme4" (Bates et al. 2015). As response variables, we used SVL and body volume, while the fixed effects we used were origin (categorical variable with three levels: Durban Rural, Durban Urban, Cape Town), age (days; continuous), and their interaction term. If the interaction term was non-significant, it was removed and the models re-run. The models also included a random intercept and slope of mesocosm ID to control dependencies in our data due to clutch effects and effects of being reared in the same mesocosm, as well as a random intercept of parentage site to accommodate dependency among observations of tadpoles with parentage from the same sampling site. To examine differences in the other variables of interest (tail length, tail muscle height, tail fin height, maximum swimming velocity, and maximum swimming acceleration), we fitted separate LMMs with the variables each using the same fixed and random effects as detailed for the growth rate and body volume models, but these models also included SVL as a fixed effect to standardise the variables with respect to body length. We did not include water temperature in any of our models examining differences in performance traits, because temperature and tadpole age are confounded. Specifically, water temperatures were lower at a higher age ($\beta = -0.033$, t = -15.76, p < .001; results from a linear model performed using the "lm" function in the R package "stats" with age as fixed effect; R Core Team 2020).

Trait differences at 40 days

We also examined trait differences at a specific point in development. We chose age 40/41 days because previous literature has shown that at this age individual traits can diverge between populations (e.g., in European common frogs, *Rana temporaria*; Van Buskirk and Arioli 2005), and also, because at this age our sample size was still relatively high (Table 1). Furthermore, metamorphosis in this species has been previously reported to commence at around an age of 40 days (du Preez et al. 2004), which also corresponded to observations made in this experiment (Suppl. material 1: Fig. S2). Once again, we fitted separate LMMs using the R package "Ime4" (Bates et al. 2015) with SVL, body volume, tail length, tail muscle height, tail fin height, maximum swimming velocity, and maximum swimming acceleration as response variables. For all models, apart from the models with SVL or body volume as response variables, we included SVL as a fixed effect. For all models, we also included origin as a fixed effect and included the random intercepts of mesocosm ID and parentage site. Again, we did not include water temperature in our models examining differences in performance variables (see above) to ensure that model results were comparable.

Developmental rate

To examine differences in developmental rate between tadpoles from different origin populations, we fitted a generalised linear mixed effects model (GLMM) with logit link function using the R package "Ime4" (Bates et al. 2015). The binomial response variable described whether tadpoles had developed to or past Gosner stage 31 (Gosner 1960) at an age of 40/41 days or not. We included origin as a fixed effect (categorical variable with three levels: Durban Rural, Durban Urban, Cape Town) and the random intercepts of mesocosm ID and parentage site.

Results

Ontogenetic differentiation of morphological and performance traits

None of the observed morphological or performance traits showed significant differences between tadpoles of different origin (Table 2, Suppl. material 1: Table S2, Fig. 2). Snout-vent length (SVL) and body volume increased with age (Table 2 and Fig. 2A, B). Tail length, tail muscle height, tail fin height, maximum swimming velocity, and maximum swimming acceleration were positively correlated with SVL (Table 2). Only tail length, relative to SVL, increased with age, whereas in relation to SVL, tail muscle height, maximum swimming velocity, and maximum swimming acceleration decreased with age (Table 2). Tail fin height, in relation to SVL, did not change with age (Table 2).

Morphological and performance trait differences at the age of 40 days

We did not find significant effects of tadpole origin on any morphological or performance trait at the age of 40/41 days (Table 3, Suppl. material 1: Table S3, Suppl. material 1: Fig. S5). Tail muscle height, tail fin height, tail length, maximum swimming velocity, and maximum swimming acceleration were all positively correlated with SVL (Table 3).

Developmental rate

The proportion of tadpoles having developed to or past Gosner stage 31 (Gosner 1960) was highest for Durban Urban (0.189), followed by Durban Rural (0.179) and was lowest for Cape Town tadpoles (0.05) as calculated from the raw data. Based on our statistical analyses, the proportion of tadpoles developing to or past Gosner stage 31 in Cape Town was only significantly lower in comparison with Durban Urban and no other significant differences were found (Table 4 and Fig. 3). This general pattern also corresponds with our data indicating that time to metamorphosis tended to be longer in Cape Town clutches than in clutches from the other habitats, although we were not able to run a statistical test on this data set due to a limited sample size (Suppl. material 1: Fig. S2).



Figure 2. Trait changes across 60 days post-hatching in tadpoles: none of the observed traits were significantly different between origins (native rural – Durban Rural, native urban – Durban Urban, non-native urban – Cape Town). Presented are **A** snout-vent length (growth rate) **B** body volume **C** tail length **D** tail muscle height **E** tail fin height **F** maximum swimming velocity, and **G** maximum swimming acceleration. All morphological and performance variables were log-transformed prior to analysis and predicted data was back-transformed before plotting. Circles represent predictions from linear mixed effect models and the lines represent predicted linear regressions with 95% confidence intervals.

Discussion

Here we show, in a common garden experiment, that the invasive urban Cape Town population of the guttural toad has a slower larval development (i.e., the proportion of tadpoles having developed to or past Gosner stage 31 at the age of 40/41 days) compared to the native urban population from Durban. Contrary to our predictions, tadpoles of urban/rural or native/invasive origin do not differ in growth rate, or any of the other morphological or performance traits we examined. From this, we suggest that prior adaptation to urban habitats (AIAI hypothesis sensu Hufbauer et al. 2012) in larval morphological, performance, and developmental traits of the guttural toad did not enhance its invasion success in Cape Town. Rather, the slower larval development in Cape Town toads arose within the short period (~ 20 years or 10 generations) since introduction, indicating bridgehead effects (e.g., Bertelsmeier and Keller 2018). Had our experiment been able to continue to metamorphosis, we would predict larger metamorphs from Cape Town given that growth rates did not differ, and which is also consistent with previous literature on the topic (e.g., Harkey and Semlitsch 1988; Newman 1989; Touchon et al. 2013; Tarvin et al. 2015). Owing to the study design used (i.e., a common garden experiment), we cannot rule out potential different habitat- or site-specific factors that may result in situational or plastic response in wild populations, however from an innate trait standpoint, we assert that the morphological



Figure 3. Probability of reaching Gosner stage 31 or higher (Gosner 1960) for tadpoles of native rural (Durban Rural), native urban (Durban Urban), and non-native urban (Cape Town) origin at an age of 40/41 days as predicted from our generalised linear mixed effect model. Presented are boxplots. The boxes denote the interquartile ranges (IQR), the bars inside the boxes correspond with the predicted medians from the models. The whiskers extend to $1.5 \times IQR$. Asterisks denote significant differences as determined by post-hoc multiple comparisons.

or performance traits we examined are not diverging within the larval stage between urban/rural or native/invasive origin populations.

One of the most prominent differences between the areas of Durban and Cape Town is the Mediterranean climate in Cape Town compared to subtropical Durban. The invasive toad population in Cape Town is confronted with a colder, drier, and more seasonal climate compared to the climate of the source habitat (Vimercati et al. 2019). Previous studies have already shown that this novel climatic regime likely led to both physiological as well as behavioural changes in adult toads (Vimercati et al. **Table 2.** Outcomes of linear mixed effect models testing for differences in morphological and performance traits between guttural toad tadpoles of native rural origin (Durban Rural), native urban origin (Durban Urban), and invasive urban origin (Cape Town) raised in a common garden environment. All variables were log-transformed prior to analysis. Presented are model coefficient estimates (β) with their corresponding standard errors (*SE*) for fixed effects and variance estimates (σ^2) for random effects and residuals. Test statistics (*t*) are given, and all significant values (p < .05) are presented in bold. For categorical variables, reference levels are presented in brackets behind the variable name.

| Model | Variable Names | Model Output | | | | |
|--|-----------------------|--------------|---------|--------|--------|--|
| Snout-Vent Length | Fixed Effects | β | SE | t | Þ | |
| C | Intercept (Cape Town) | 0.412 | 0.042 | 9.740 | .011 | |
| | Origin (Durban Rural) | 0.064 | 0.052 | 1.239 | .341 | |
| | Origin (Durban Urban) | 0.025 | 0.052 | 0.492 | .672 | |
| | Age | 0.006 | < 0.001 | 11.970 | < .001 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | 0.002 | | | | |
| | Residuals | 0.008 | | | | |
| Body Volume | Fixed Effects | β | SE | t | p | |
| | Intercept (Cape Town) | 1.208 | 0.151 | 7.983 | .016 | |
| | Origin (Durban Rural) | 0.220 | 0.186 | 1.187 | .358 | |
| | Origin (Durban Urban) | 0.114 | 0.185 | 0.614 | .602 | |
| | Age | 0.020 | 0.002 | 12.606 | < .001 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | 0.026 | | | | |
| | Residuals | 0.085 | | | | |
| Tail Muscle Height | Fixed Effects | β | SE | t | Þ | |
| Body Volume Tail Muscle Height Tail Fin Height Tail Length | Intercept (Cape Town) | -0.544 | 0.035 | -5.690 | .003 | |
| | Snout-Vent Length | 0.542 | 0.017 | 31.681 | < .001 | |
| | Origin (Durban Rural) | -0.013 | 0.042 | -0.306 | .789 | |
| | Origin (Durban Urban) | 0.002 | 0.042 | 0.041 | .971 | |
| | Age | -0.003 | < 0.001 | -5.578 | < .001 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | 0.001 | | | | |
| | Residuals | 0.008 | | | | |
| Tail Fin Height | Fixed Effects | β | SE | t | Þ | |
| Tail Muscle Height Tail Fin Height Tail Length | Intercept (Cape Town) | -0.009 | 0.064 | -0.139 | .902 | |
| | Snout-Vent Length | 0.393 | 0.012 | 31.917 | < .001 | |
| | Origin (Durban Rural) | -0.049 | 0.078 | -0.577 | .623 | |
| | Origin (Durban Urban) | -0.040 | 0.078 | -0.517 | .657 | |
| | Age | -0.004 | 0.009 | -0.434 | .670 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | 0.004 | | | | |
| | Residuals | 0.004 | | | | |
| Tail Length | Fixed Effects | β | SE | t | Þ | |
| 0 | Intercept (Cape Town) | 0.265 | 0.009 | 29.937 | < .001 | |
| | Snout-Vent Length | 0.729 | 0.011 | 68.726 | < .001 | |
| | Origin (Durban Rural) | 0.009 | 0.010 | 0.992 | .437 | |
| | Origin (Durban Urban) | 0.014 | 0.009 | 1.522 | .290 | |
| | Age | 0.001 | < 0.001 | 6.995 | < .001 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | < 0.001 | | | | |
| | 0 | | | | | |

| Model | Variable Names | Model Output | | | | |
|-------------------------------|-----------------------|--------------|---------|--------|--------|--|
| | Residuals | 0.003 | | | | |
| Maximum Swimming Velocity | Fixed Effects | β | SE | t | Р | |
| | Intercept (Cape Town) | 1.529 | 0.090 | 17.066 | .003 | |
| | Snout-Vent Length | 0.667 | 0.026 | 25.656 | < .001 | |
| | Origin (Durban Rural) | 0.030 | 0.109 | 0.277 | .808 | |
| | Origin (Durban Urban) | 0.046 | 0.109 | 0.423 | .714 | |
| | Age | -0.004 | 0.001 | -3.348 | .004 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | 0.008 | | | | |
| | Residuals | 0.018 | | | | |
| Maximum Swimming Acceleration | Fixed Effects | β | SE | t | P | |
| | Intercept (Cape Town) | 2.819 | 0.042 | 66.838 | < .001 | |
| | Snout-Vent Length | 0.592 | 0.032 | 18.303 | < .001 | |
| | Origin (Durban Rural) | 0.056 | 0.049 | 1.142 | .374 | |
| | Origin (Durban Urban) | 0.051 | 0.049 | 1.036 | .414 | |
| | Age | -0.001 | < 0.001 | -2.505 | .017 | |
| | Random Effects | σ^2 | | | | |
| | Mesocosm ID | < 0.001 | | | | |
| | Parentage Site | 0.002 | | | | |
| | Residuals | 0.028 | | | | |

2018; Vimercati et al. 2019; Madelaire et al. 2020; Barsotti et al. 2021). For example, Madelaire et al. (2020) demonstrated that Cape Town adults show a more efficient water-conservation behaviour than toads from Durban. From extensive research on the effects of toad metamorph size, we know that larger metamorphs desiccate less rapidly and, are able to disperse further, potentially increasing chances of reaching new bodies of water (Cohen and Alford 1993; Goater et al. 1993; Beck and Congdon 2000; Chelgren et al. 2006; Child et al. 2008; Cabrera-Guzmán et al. 2013). Thus, the drier summers in Cape Town, during which guttural toads metamorphose, may lead to strong selection for larger metamorph body sizes (as we hypothesize will arise via the longer larval developmental time found in this study) in the invasive population.

Given the substantial literature reporting differences in tadpole growth rates due to ecological or evolutionary factors, we were surprised that we did not find any differences in growth rate among the three location types. Several studies on body size differences among rural and urban, as well as among native and invasive populations, across a wide range of taxa report higher growth rates and larger adult body sizes for urban and/or invasive populations (Phillips 2009; y Gomez and Van Dyck 2012; Sargent and Lodge 2014; Hall and Warner 2017; Iglesias-Carrasco et al. 2017; Pujol-Buxó et al. 2020; Putman and Tippie 2020), which is widely attributed to lower interspecific competition, higher food abundance, and novel ecological opportunities in urban and/ or invasive ranges. The opposite trend is, however, also possible. For example, studies of cladocerans, several orders of insects, and on passerine birds (Brans et al. 2017a, b; Gianuca et al. 2018; Merckx et al. 2018; Corsini et al. 2021) have found negative effects of urban environments on body size. In the case of our study species, the guttural toad, Baxter-Gilbert et al. (2020) showed that invasive populations of guttural toads **Table 3.** Model output of linear mixed effect models examining differences in guttural toad tadpoles (*Sclerophrys gutturalis*) at the age of 40/41 days between guttural toad tadpoles of native rural origin (Durban Rural), native urban origin (Durban Urban), and invasive urban origin (Cape Town) raised in a common garden environment. All morphological and performance variables were log-transformed prior to analysis. Given are model coefficient estimates (β) with their corresponding standard errors (*SE*) for fixed effects and variance estimates (σ^2) for random effects and residuals. Test statistics (t) are presented, and all significant values (p < .05) are presented in bold. For categorical variables, reference levels are presented in brackets behind the variable name.

| Model | Variable Names | Model Output | | | |
|---------------------------|-----------------------|--------------|-------|--------|--------|
| Snout-Vent Length | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | 0.659 | 0.026 | 25.291 | < .001 |
| | Origin (Durban Rural) | 0.025 | 0.032 | 0.796 | .437 |
| | Origin (Durban Urban) | 0.043 | 0.031 | 1.362 | .190 |
| | Random Effects | σ^2 | | | |
| | Mesocosm ID | 0.002 | | | |
| | Parentage Site | 0.000 | | | |
| | Residuals | 0.006 | | | |
| Body Volume | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | 1.993 | 0.075 | 26.487 | < .001 |
| | Origin (Durban Rural) | 0.070 | 0.092 | 0.757 | .459 |
| | Origin (Durban Urban) | 0.141 | 0.090 | 1.564 | .135 |
| | Random Effects | σ^2 | | | |
| | Mesocosm ID | 0.020 | | | |
| | Parentage Site | 0.000 | | | |
| | Residuals | 0.051 | | | |
| Tail Muscle Height | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | -0.684 | 0.080 | -8.572 | .004 |
| | Snout-Vent Length | 0.659 | 0.050 | 13.212 | < .001 |
| | Origin (Durban Rural) | -0.010 | 0.089 | -0.113 | .921 |
| | Origin (Durban Urban) | 0.029 | 0.089 | 0.327 | .775 |
| | Mesocosm ID | 0.001 | | | |
| | Parentage Site | 0.005 | | | |
| | Residuals | 0.007 | | | |
| Tail Fin Height | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | -0.038 | 0.025 | -1.525 | .147 |
| | Snout-Vent Length | 0.456 | 0.031 | 14.914 | < .001 |
| | Origin (Durban Rural) | -0.024 | 0.018 | -1.363 | .315 |
| | Origin (Durban Urban) | -0.018 | 0.018 | -1.032 | .424 |
| | Random Effects | σ^2 | | | |
| | Mesocosm ID | < 0.001 | | | |
| | Parentage Site | < 0.001 | | | |
| | Residuals | 0.002 | | | |
| Tail Length | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | 0.312 | 0.023 | 13.772 | < .001 |
| | Snout-Vent Length | 0.734 | 0.031 | 23.409 | < .001 |
| | Origin (Durban Rural) | 0.014 | 0.011 | 1.220 | .238 |
| | Origin (Durban Urban) | 0.017 | 0.011 | 1.534 | .142 |
| | Random Effects | σ^2 | | | |
| | Mesocosm ID | < 0.001 | | | |
| | Parentage Site | 0.000 | | | |
| | Residuals | 0.003 | | | |
| Maximum Swimming Velocity | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | 1.329 | 0.089 | 14.999 | < .001 |
| | Snout-Vent Length | 0.754 | 0.075 | 10.012 | < .001 |
| | Origin (Durban Rural) | -0.007 | 0.090 | -0.083 | .942 |
| | Origin (Durban Urban) | 0.078 | 0.090 | 0.865 | .479 |

| Model | Variable Names | Model Output | | | |
|-------------------------------|-----------------------|--------------|-------|--------|--------|
| | Random Effects | σ^2 | | | |
| | Mesocosm ID | < 0.001 | | | |
| | Parentage Site | 0.005 | | | |
| | Residuals | 0.018 | | | |
| Maximum Swimming Acceleration | Fixed Effects | β | SE | t | P |
| | Intercept (Cape Town) | 2.675 | 0.110 | 24.272 | < .001 |
| | Snout-Vent Length | 0.737 | 0.099 | 7.421 | < .001 |
| | Origin (Durban Rural) | 0.008 | 0.109 | 0.077 | .945 |
| | Origin (Durban Urban) | 0.112 | 0.109 | 1.028 | .413 |
| | Random Effects | σ^2 | | | |
| | Mesocosm ID | 0.001 | | | |
| | Parentage Site | 0.007 | | | |
| | Residuals | 0.029 | | | |

on the islands of Mauritius and Réunion have smaller adult body sizes compared to native populations. Observations made in this study (see Suppl. material 1: Fig. S1) indicate that adult body sizes might diverge between rural, urban, and invasive populations, as well. Yet, our study suggests that any adult body size differences are not driven by innate differences in tadpole growth rate, and it is unclear how they arose in adults (e.g., due to environmental factors such as food, due to phenotypic plasticity or due to directional selection for smaller or larger adult body sizes). In another successful amphibian invader, the African clawed frog (Xenopus laevis), Kruger et al. (2021) showed that despite differences in adult body sizes between the core and periphery of the species' invasive distribution in France, the tadpoles did not show significant differences in growth rates when raised in a common garden experiment, indicating the decoupling of traits between life stages (but see Phillips 2009 for coupling of growth rate across larval and juvenile stages in cane toads, Rhinella marina). The decoupling of traits might also be a potential driver for the apparent similarities in growth rates of guttural toad tadpoles. Furthermore, the guttural toad is an infamous urban exploiter with synanthropic behaviour, frequently making use of artificial light sources for foraging and artificial bodies of water for hydration and reproduction (du Preez et al. 2004; Measey et al. 2017; Vimercati et al. 2017; Baxter-Gilbert et al. 2020; Measey et al. 2020b). Thus, the terrestrial stage of the species might benefit from ecological opportunities in urban environments that are not available for the aquatic stage and growth rate might be decoupled between the tadpole and adult stage.

Urban bodies of water have been reported to frequently show high levels of modification (especially of the riparian zone) and, partly as a consequence, show reduced native biodiversity and high abundance of invasive species (reviewed in Hassall 2014), but can also act as biodiversity hotspots in a city (Hassall 2014; Hill et al. 2017a). Given the potential reduction in predation and competition for Durban Urban and Cape Town tadpoles, we expected that Durban Rural tadpoles would show the most conducive phenotype for predator escape and overall better performance under high competition scenarios (i.e., high burst escape swimming speed, slim bodies and large tail fins; Smith and Van Buskirk 1995; Relyea 2002; Relyea 2004; Dayton et al. 2005), which is in line with the enemy release hypothesis (Colautti et al. 2004; Liu and Stiling **Table 4. (A)** Model output of a generalised linear mixed effects model used to examine differences in guttural toad (*Sclerophrys gutturalis*) tadpole developmental rates across origins. We present model coefficient estimates (β) and the corresponding standard errors (*SE*) for fixed effects, as well as variance estimates (σ^2) for random effects. Test statistics (*z*) are presented and all significant values (p < .05) are presented in bold. For the categorical variables, reference levels are presented in brackets behind the variable name. (**B**) Results of post-hoc multiple comparisons testing for differences in developmental rate among guttural toad (*Sclerophrys gutturalis*) tadpole origins. Presented are conditional odds ratios with their corresponding standard errors (*SE*). Test statistics (*z*) and *p*-values (p_{corr}) corrected using a "tukey" adjustment (Lenth et al. 2018) are given. Significant values are bolded.

| | (A) Output from the generalised linear mixed effect model | | | | |
|-----------------------------|---|---------------------------------|------------|--------|------------|
| Variable Names | | | | | |
| Fixed Effects | | β | SE | z | Р |
| Intercept (Cape Town) | | -3.08 | 0.599 | -5.142 | < .001 |
| Origin (Durban Rural) | | 1.37 | 0.663 | 2.066 | .039 |
| Origin (Durban Urban) | | 1.534 | 0.654 | 2.347 | .019 |
| Random Effects | | σ^2 | | | |
| Mesocosm ID | | 0.324 | | | |
| Parentage Site | | < 0.001 | | | |
| | | (B) Multiple Comparisons betwee | en Origins | | |
| Origin Comparison | | Conditional Odds Ratio | SE | z | P_{corr} |
| Cape Town – Durban Rural | | 0.254 | 0.169 | -2.066 | 0.097 |
| Cape Town – Durban Urban | | 0.216 | 0.141 | -2.347 | 0.049 |
| Durban Rural – Durban Urban | | 0.849 | 0.344 | -0.404 | 0.914 |

2006). However, similar to growth rate, swimming performance and the underlying morphological variables did not diverge between tadpoles of different origin. The urban (Durban Urban) and non-native urban (Cape Town) sampling sites frequently had artificial ponds that were used for breeding by guttural toads (Vimercati et al. 2017) and, also frequently contained non-native ornamental fish species such as goldfish (Carassius auratus) and koi (Cyprinus rubrofuscus) (MM, JB-G & JM pers. obs.). Similarly, the water bodies in our rural sampling sites also contained fish, but of native fish species (MM, JB-G & BM pers. obs.) Ornamental fish species as well as many fish species, native to sub-tropical South Africa, are well-known to reduce invertebrate densities such as dragonfly larvae (reviewed in Wellborn et al. 1996; Brown et al. 2012; see also Richardson et al. 1995; Preston et al. 2017) but may not predate on toad tadpoles due to unpalatability (see Brown et al. 2012 and citations within; Manteifel and Reshetnikov 2002; Üveges et al. 2019). Invertebrate predators, on the other hand, have been reported to reduce densities of the larvae of some invasive amphibian species, like cane toads (Crossland and Alford 1998; Cabrera-Guzmán et al. 2012) and African clawed frogs (Warren et al. 2021). Thus, ornamental fish species may carry out a similar ecological function as native fish species and therefore, a similar selection regime is present in both rural and urban ponds from the perspective of guttural toads. More research is needed, however, to understand the facilitating role ornamental and/ or invasive fish species might play in urban bodies with respect to the invasion success of pond-breeding amphibians (e.g., Adams et al. 2007).

In this common garden experiment, we raised the F1 progeny from toads collected in the wild. Thus, we cannot rule out maternal/paternal effects on differences or similarities between tadpoles (e.g., Kawecki and Ebert 2004), and the effect of phenotypic plasticity induced by environmental cues in the tadpole's respective habitats that might enhance fitness under the current selective regime. Anuran tadpoles are well-known to show high levels of phenotypic plasticity in swimming performance and underlying morphological traits in response to a variety of environmental factors (e.g., Loman and Claesson 2003; Relyea 2004; Hoverman et al. 2005; Castaneda et al. 2006; Whiles et al. 2010; Gomez-Mestre et al. 2013). Interestingly, Bókony et al. (2021) demonstrated that urban tadpoles of common toads (Bufo bufo) showed higher baseline stress and a more efficient recovery to baseline levels compared to tadpoles from natural habitats, partly as a response to water pollutants. These differences disappeared, however, when individuals were raised in a common garden environment, suggesting that divergence in the efficacy of negative feedback is driven by phenotypic plasticity rather than microevolutionary change (Bókony et al. 2021). Urban-exploitative and invasive species often express highly flexible generalist phenotypes that enable them to colonise new habitats such as cities (Rodewald and Gehrt 2014; Ducatez et al. 2018; Franzén et al. 2020; Palacio 2020) and phenotypic plasticity plays an important role in facilitating the success of a small number of highly abundant species in cities (reviewed in Lowry et al. 2013; Rodewald and Gehrt 2014). Therefore, phenotypic flexibility might also play an important role in the invasion success of guttural toads and we suggest future research to investigate if and how phenotypic plasticity might drive invasion success in this and other urban adapted species.

We show here that prior adaptation in larval growth rate as well as morphological and performance traits is unlikely to have facilitated the invasion success of guttural toads in Cape Town. Furthermore, the reduction in developmental rate likely arose after the introduction to Cape Town. Thus, bridgehead effects and decoupled evolution of traits are more likely to drive successful colonisation of new habitats in this species. Our findings suggest several promising avenues of future research. For example, we suggest investigations examine divergent selection for aquatic and terrestrial life stages in amphibian invaders, and how this might lead to coupling or decoupling of traits across life stages. We also know little about how certain habitat characteristics in urban environments, such as altered species composition or anthropogenic structures, might facilitate colonisation of invasive populations or how possible plastic traits can enhance invasion success across different habitat types - which are both important knowledge gaps to address.

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Supplementary material I

Supporting information

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Data type: text file

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Corythucha arcuata (Say, 1832) (Hemiptera, Tingidae) in its invasive range in Europe: perception, knowledge and willingness to act in foresters and citizens

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Abstract

The oak lace bug (OLB) *Corythucha arcuata* (Say, 1832) is an invasive alien species (IAS) that potentially could have many negative impacts on European oak health. Certain measures can be applied to counteract these effects. However, these measures may not be acceptable for forest managers or other stakeholder groups, such as private forest owners, environmental NGOs or the general public. Thereby, we set out to study the perception and knowledge of foresters and other stakeholders on the health status of European oak forests affected by oak lace bug and to investigate what forest health management measures would be acceptable to these target groups. An online survey questionnaire was designed and distributed via social

networks, as well as professional networks via e-mails. The survey questionnaire was completed by 2084 respondents from nine European countries: Austria, Croatia, Belgium, France, Hungary, Italy, Romania, Serbia and Slovenia. Even though only a little over 60% of respondents reported they had noticed the discolouration of oak leaves caused by OLB, almost all (93%) considered it to be a problem. As respondents come from a country where *C. arcuata* is widespread and established, people's general knowledge and awareness of OLB began to increase. The survey revealed that foresters thought that the insect affected photosynthesis, acorn crop and the aesthetics of the trees, but cannot cause death of trees. However, they assume that the value of the wood would decrease (this fact is also supported by the respondents who are connected to an environmental NGO), but that OLB does not affect property value. However, forest owners claim that the value of the property can be affected and that people would avoid entering the forest. In terms of potential control methods, respondents preferred biological or mechanical measures over chemical ones. We consider this study to be a good basis for further research on the topic of perception, knowledge and attitudes related to OLB since we can expect that the IAS, such as OLB, will certainly spread to European countries that were not included in this survey.

Keywords

Attitude, citizen knowledge, Europe, forest health, IAS control measures, invasive alien species, survey

Introduction

Humans rely on healthy forest ecosystems to provide a wide range of ecosystem services (Trumbore et al. 2015). Furthermore, in the Sustainable Development Goals Report (2020), the United Nations have set two goals (13-Climate action and 15-Life on land) to the expansion of sustainable forest management to protect biodiversity and ecosystems in order to strengthen resilience and the capacity to adapt to climate risks. Assuming that trees are key components of forest ecosystems (Turner and Daily 2008; Bateman et al. 2013), the way in which forest ecosystem services are provided is strongly influenced by the health of these trees. However, various tree pests and disease threaten the health of forest ecosystems (Boyd et al. 2013). Although native forests are adapted to a certain level of disturbance, in addition to problems such as climate change or air pollution, more and more forests are also facing invasive alien species (IAS) (Trumbore et al. 2015). Invasive alien species are organisms that have been introduced by humans out of their natural environment, either deliberately or accidentally, have established and subsequently multiplied and, thus, begun to have negative effects on the newly-invaded ecosystem (Williamson and Fitter 1996; Juliano and Philip Lounibos 2005; EEA 2013).

Negative effects of invasive alien species can be particularly profound in long-lived ecosystems, such as forests (Régnière 2011). They can cause multiple consequences on the environment, the economy or even human health (Pimentel et al. 2000; Lovell et al. 2006; Meyerson and Mooney 2007; Vilà et al. 2010, 2011; Jeschke et al. 2013; Simberloff et al. 2013; Blackburn et al. 2014; Hulme 2014; Schindler et al. 2015). As a result of the increase in transport activity, human trade and the anthropogenic influence on the climate, forest ecosystems are increasingly disrupted by biological

invasions (Régnière 2011). Until recently, invasive arthropods have been given less attention compared to other invasive organisms, such as plants, vertebrates or aquatic species (Kenis et al. 2009). In Europe, the rate of reports of invasive alien insect species has almost doubled in recent decades and currently insects represent 87% of invasive alien species on the European continent (Roques 2010).

Corythucha arcuata, the oak lace bug (OLB henceforth), is an insect from North America that was first reported in Europe in 2000 in northern Italy (Bernardinelli and Zandigiacomo 2000). Up until 2019, it had been detected in 20 European countries (Paulin et al. 2020). The insect causes discolouration of the foliage of the host trees (mainly *Quercus* spp.) caused by feeding of both nymphs and adults on the underside of the leaves. As oak ecosystems are extremely important both from an economic and ecological point of view in Europe, the species raised significant awareness. Recognising its potential degree of injury and invasion, the OLB was included on the EPPO Alert List in March 2001 and remained so until 2007, when it became clear that phytosanitary efforts could not stop its expansion (EPPO 2001, 2007). Furthermore, C. arcuata has also been suspected to be a nuisance as the adults may sting as was shown for C. ciliata (Say, 1832) (Dutto and Bertero 2013). However, this should not be confused with an aggressive behaviour like the stinging done by wasps. The real explosive expansion in Europe was observed only a decade after the first report, with the estimated total area of forests infested by OLB in only five countries (Croatia, Hungary, Romania, European part of Russia and Serbia) exceeding 1.7 million hectares in 2019 (Paulin et al. 2020).

Mechanical control measures have been tested in several countries, such as the UK, Czech Republic and Romania (Williams et al. 2021), using yellow sticky traps or suction traps. However, the methodologies evaluated were more for early detection, surveying and monitoring and not for removal. For biological control to be effective, identification of suitable biotic factors are needed. Previous studies conducted on identifying potential predators and fungal pathogens have identified several species (Coccinellidae, Chrysopidae, mites, spiders, Beauveria bassiana) that can reduce insect numbers at different stages of development (Bernardinelli and Zandigiacomo 2000; Sönmez et al. 2016; Kovač et al. 2020; Paulin et al. 2020). Nevertheless, these studies have shown that natural enemies in Europe that might control OLB populations are not having any effects. Another proposed method of biological control could be to use a classical biological control programme against OLB in Europe that may be achieved by importing natural enemies of OLB from North America (Puttler et al. 2014). The use of the latter method will have to be carefully chosen and managed with discernment, because the introduction of an allogeneic species into Europe, even for biocontrol purpose, could have further negative impacts. A potential chemical control option was researched in a Romanian study in two isolated forests (Bălăcenoiu et al. 2021). The results showed that after more or less time (earlier to the contact insecticide and later to the systemic one), the treated forests were re-infested by OLB; hence, it could not be economically justified, especially given the large area of infested European forests. However, recommending the use of this control method in isolated cases, such as frequented parks, tourist attractions, gardens and park forests, may be possible, in

view of limiting the discomfort caused by the OLB stings/bites on the human skin (Ciceoi and Radulovici 2018), depending whether insecticides are allowed in these areas, especially in urban areas.

In the last three decades, out of 77 studies that analysed social perception of invasive alien species, only 13 targeted the taxonomic group of insects (Kapitza et al. 2019). This means that we still have little information on whether and how the public perceive invasive alien insects and, in particular OLB, as well as what kind of management of these alien insects would be acceptable to the general public. A recent study showed that people generally support management of invasive species (Japelj et al. 2019). However, there is no such study targeting management of OLB.

The objectives of our study were to investigate perception and knowledge of the health status of European oak forests affected by OLB as perceived by several stake-holder groups (foresters, private forest owners, environmental NGOs and the general public), as well as to explore attitudes of these stakeholder groups towards actual and potential measures targeting OLB. We hypothesised that: a) selected stakeholder groups perceive OLB or, at least, the effect of OLB on oak species, to some extent, b) that attitude of stakeholder groups towards possible measures for OLB management may differ and c) that the sociodemographic characteristics of respondents may be significantly connected to their perception, knowledge and attitudes. We expected differences in perception and knowledge between respondents from countries where OLB has been already established and those where it is still not present. When it comes to attitudes towards specific measures for controlling OLB, we also expected that support of different stakeholder and sociodemographic groups may differ.

Materials and methods

The questionnaire was designed first in English (Suppl. material 1: Appendix 1) and then it was translated into several local languages to be distributed in European countries that showed a gradient of invasion of OLB: not occurring (absent: Belgium, United Kingdom), arrived not more than four years ago (spread: Slovenia, Austria, France) and invaded for more than 5 years (established: Croatia, Hungary, Italy, Romania, Serbia). For each country, there was at least one national contact person, who translated the questionnaire article from English into the local language. As the questionnaire distribution was made exclusively in digital form for each language, the questionnaire was uploaded to the Google Forms platform and continued to be computer-assisted web interview (CAWI method) until the study was completed. We used a snowball approach to distribute the questionnaire, sending it to relevant academic and professional contacts through mailing lists and advertised it on social media, such as Facebook, LinkedIn and WhatsApp. The survey was conducted in September and October 2020, when the effects of OLB were most easily noticed by the general public.

We aimed to reach particular target groups – foresters, environmental activists, nature lovers, forest owners and members of environmental NGOs, as well as the

general public. During the period the questionnaire was accessible, we surveyed the self-declared pre-defined categories of respondents and re-advertised the questionnaire through the relevant channels to reach under-represented groups.

Questionnaire design

The questionnaire did not focus exclusively on OLB, but addressed some issues generally related to invasive alien species and was split into three parts, namely: 1) questions on invasive species in general, 2) a section that was specifically dedicated to the issue, knowledge and perception of the OLB in European oak forests and 3) a part with questions on respondents' socio-demographic characteristics. In this paper, we will present results related to OLB and sociodemographic characteristics of respondents.

The questionnaire was anonymous. There were no means by which respondents could be identified from their answers. By submitting their answers, respondents were informed that they will be analysed and used in the context of a research project, leading to publication in a scientific journal.

The questions were mostly a close-ended format, with several types of answers: binary answer (yes or no), multiple possible answers, Likert scale or open-ended. However, the open-ended questions (in which the respondent could give their own opinion) yielded a small number of responses and they were very diverse. We, therefore, did not perform any quantitative analysis, but instead extracted and synthesised the most frequent answers to be presented here.

Data analysis

To study the influence of OLB's time since introduction on respondents' perception and general knowledge of OLB and attitudes towards its management, the countries were grouped as described above into "not arrived", "recently arrived" and "present for greater than 5-years" categories. After the questionnaire was distributed within 10 European countries, given that we only received 15 responses from the United Kingdom, this information was removed from any further data analysis.

We analysed closed 'yes-no' questions (questions 1, 2, 4 and 6–8) using generalised linear mixed models with binomial error distribution and logit link and with country included as a random effect. The questions 9–10 (multiple possible answers) were analysed with an ordinal mixed model, with again country included as a random effect. We first built a full model including the following independent variables (Suppl. material 2: Appendix 2) as fixed effects: whether respondents self-identified as foresters (yes/no), landowners (yes/no), being connected to an environmental NGO, time spent in the forest (frequency of forest visit), gender, age and time since OLB introduction in the country (absent, spread and established). We then compared the full model with every possible model through a model selection procedure based on the Akaike's Information Criterion (AIC). The model with the lowest AIC is generally considered the best model, given the data and set of candidate models. Every model within 2 units is AIC units from the best model is considered as equivalent in their ability to fit the data.

Analysis and data visualisation were carried on using the R statistical programme (R Core Team 2020), with the packages "MASS", lme4 (Bates et al. 2015), (Venables and Ripley 2002), ggalluvial (Brunson and Read 2020) and "ordinal" (Christensen 2019) being used.

Results

Finally, in the nine remaining countries, the questionnaire was completed by 2084 respondents. Regarding socio-demographic characteristics, the questionnaire revealed respondents were as follows: foresters/non-foresters 37%/63%, forest owners/non-forest owners 21%/79%, environmental NGOs/non-NGOs 31%/69%, women/men 37%/63%.

I. General knowledge of respondents about OLB

Based on photos representing whole trees or single leaves impacted by the OLB, two thirds of respondents (66%) declared that they had seen such discolouration before (Fig. 1). Respondents who were foresters (z = 2.108, P = 0.030), connected to an environmental NGO (z = 2.877, P = 0.004) and frequent visitors of the forest especially individuals who went weekly (z = 2.347, P = 0.019) were more likely to respond



Figure 1. The extent to which people had seen the discolouration before the survey.



Figure 2. The extent to which respondents consider discolouration to be a problem.

that they had seen the discolouration before. In addition, the probability that the respondents from countries where OLB had been present for a while (established group) were more likely to have previously seen the discolouration compared to countries without OLB (z = 2.356, P = 0.018).

Almost all respondents (93%) consider this discolouration to be a problem, with frequent forest visitors and women being more likely to hold this opinion (Fig. 2).

In order to determine to what extent the respondents knew the cause of the discolouration, we listed several potential causal biotic and abiotic factors and offered them the possibility of a single answer. Only 5% of respondents perceived the colour of leaves normal given the season. Of the 95% of the respondents who recognised discolouration (i.e. abnormal leaf colour), 41% assumed it was caused by an insect, 36% by a pathogen, 14% attributed it to drought and 4% gave open answers (many respondents associated discolouration with pollution or climate change).

After we presented them with a picture with OLB, indicating that it had caused the discolouration, we asked if they had seen this insect before – half of the respondents declared having seen the insect before (Fig. 3). Forest managers (z = 7.422, P = 1.15e-13), frequent forest visitors (once a day: z = 2.824, P = 0.005; once a week: z = 2.335, P = 0.020), younger people (18–25 years) compared to mature and older respondents (46–55 years: z = -4.035, P = 5.46e-0; 56–65 years: z = -4.327, P = 1.51e-05; more than 65 years: z = -5.988, P = 2.12e-09) or those from countries where OLB had been present for a while (established group countries)



Figure 3. The extent to which people had seen the insect that causes leaf discolouration before the survey.

(established group: z = 4.682, P = 2.84e-06) were more likely to have seen the insect before (Fig. 3).

Respondents were further asked if they knew the name of the insect species they had seen or the one causing the leaf discolouration and offered them several responses. Most of the respondents correctly identified *Corythucha arcuata* (50%) or acknowledged their inability to recognise the species (42%). The rest of the respondents offered predefined answers, such as *Corythucha ciliata*, *Cameraria ohridella* Deschka & Dimić, 1986 or one of the native insect species.

We then informed our respondents that the correct answer was *Corythucha arcuata* (OLB) and asked them if they had ever heard of this species before. Half of respondents (51%) replied "yes" to this question (Fig. 4). More than three-quarters (77%) of those who said they had heard of this species before recognised the species in the previous question. Being a forest manager (Fig. 6B) (z = 9.305, P = < 2e-16), not a forest owner (z = -2.125, P = 0.034) or connected to an environmental NGO (passively: z = 2.087, P = 0.037; actively: z = 4.149, P = 3.33e-05) increased the likelihood that they had heard about *Corythucha arcuata* before the survey. Additionally, young respondents (18–25 years), compared to mature and older respondents (46–55 years: z = -2.556 P = 0.011; 56–65 years: z = -1.934, P = 0.053; more than 65 years: z = -2.696, P = 0.007), more frequent forest visitors (once a day: z = 4.368, P = 1.25e-05; once a week: z = 3.109, P = 0.002; once a month: z = 2.199, P = 0.028) and respondents who came from countries where OLB had already been reported (established group compared to absent group: z = 3.900, P = 9.61e-05) were also more likely to have heard about the species (Fig. 4).



Figure 4. The extent to which respondents had heard about *Corythucha arcuata* after the species' name was mentioned in the questionnaire.

2. Perception of respondents about OLB effects on affected trees

Parameter estimates suggest that forest managers are of the opinion that even if OLB affects photosynthesis of the trees, it cannot cause their death in time, but it affects the aesthetics of the trees. None of the respondents considers that the insect has no effects on the trees. Forest owners (z = 2.489, P = 0.013) and younger people (older than 65 years: z = -2.580, P = 0.010) thought it affects the aesthetics of the trees. The likelihood that respondents are of opinion that OLB causes the die-off of infested trees in time was higher in countries where OLB already occurred (recent occurrence: z = 2.583, P = 0.010; established: z = 4.349, P = 1.37e-05) and higher amongst non-foresters (z = -4.675, P = 2.94e-06).

People who were not foresters (z = -2.065, P = 0.039), those who visit forests very rarely (once a day: z = -2.627, P = 0.009, once a week: z = -1.947, P = 0.052, once a month: z = -2.546, P = 0.011, once a year: z = -2.472, P = 0.013) and those who come from the countries where OLB had not yet been reported prior to the survey or only was established recently (spread group: z = -0.680, P = 0.496; established group: z = -3.020, P = 0.002), did not know what effects OLB might have on affected trees.

Respondents who are actively participating in an environmental NGO (z = 2.409, P = 0.016) and men (z = 2.517, P = 0.012) felt the need to add other effects besides the

predefined list. They answered mainly in the same direction, highlighting that OLB was likely to affect oak growth, decrease the acorn crop or weaken the resistance system of trees to other factors.

3. Perception of respondents to the effects of OLB on society

Parameter estimates suggest that women (compared to men: z = -2.700, P = 0.007) or active members of an environmental NGO (z = 2.317, P = 0.02049) are more likely to be of the opinion that one of the impacts of OLB would be to decrease the value of wood, while forest managers are more likely to be of the opinion that OLB does not affect property value (z = -2.689, P = 0.007158). In addition, older people (z = 2.379, P = 0.0174) or people from established group countries, where OLB has been established longer (z = 2.297, P = 0.0216), are of the opinion that one of the effects would be that people will avoid entering the forest. Women (compared to men: z = -4.405, P = 1.06e-05) or young people (compared to mature people between 46 and 55: z =-2.679, P = 0.00739; between 56 and 65: z = -3.203, P = 0.00136), are more likely to consider that an important effect on society caused by OLB is the discomfort caused by the insect's stings/bites on the human skin.

It was statistically significant that respondents from countries where OLB has not arrived or recently arrived (absent and spread group), in comparison to where OLB has been longer established, oppose the idea that it does not have any effect on society (z = -3.843, P = 0.000122). People who are less than once per year in the forest did not know whether this affects society compared to people who are relatively frequent visitors in the forest (once a month: z = -2.441, P = 0.0147). The same is observed for people living in areas without OLB (absent group) compared to areas where OLB has been established for a while (z = -3.992, P = 6.55e-05). In addition, respondents from countries where OLB occurs (spread group: z = -2.029, P = 0.04251, established groups: z = -2.344, P = 0.01907), forest managers (z = 2.359, P = 0.01832), people who are actively participating in an environmental NGO (z = 2.331, P = 0.01974) and people in the age class between 36 and 65 years (compared to people older than 65: 36–45 years: z = 2.804, P = 0.00505, 46–55 years: z = 2.437, P = 0.01482; 56–65 years: z = 2.889, P = 0.00386) completed the questionnaire citing other effects that were not in the predefined list of answers. Most answers were similar and were generally focused on similar themes as potential negative impacts, such as economic costs for the owner, increasing CO₂, ecological imbalance and loss of social function of forests.

4. Attitude of respondents towards control methods of OLB

When asking respondents how strongly they would support removal of OLB to a certain extent, their attitudes towards certain measures differed. The respondents were more likely to support partial removal for the purpose of preventing further spread (Fig. 5) than complete removal for the purpose of total eradication (Fig. 6). Almost three quarters (72%) supported partial removal to varying degrees (mostly support/fully



Figure 5. Attitudes of respondents towards the partial removal of oak lace bug (OLB). 0 = I do not support; 1 = I partially support; 2 = I mostly support; 3 = I fully support.



Figure 6. Attitudes of respondents towards the complete removal of OLB. 0 = I do not support; 1 = I partially support; 2 = I mostly support; 3 = I fully support.



Figure 7. The influences of socio-demographic characteristics (foresters/other, environmental NGO/ other) on support for mechanical control measures. 0 = I do not support; 1 = I partially support; 2 = I mostly support; 3 = I fully support.

support), while two thirds (64%) supported complete removal to a greater or lesser degree (mostly support/fully support). At the same time, 13% of respondents did not support complete removal and partial removal was not supported by 9% of respondents.

Regarding the significant influence of socio-demographic characteristics, the results were more complicated. It appeared that, with increasing age (although not significantly), respondents were more likely not to support the removal of OLB. Men (compared the women: z = 2.750, P = 0.005956) or those who were between 36 and 45 years old (compare to people older than 65 years: z = 3.853, P = 0.000117) were more likely to support complete removal.

When asked how strongly they would support various removal measures, it was obvious that respondents preferred biological or mechanical control measures (69% mostly and fully support), over chemical ones with only 9% of the respondents who did not support mechanical removal. The degree of support for chemical control measures was more balanced, with 48% supporting them (full support: 9%, partial support: 39%), whereas 39% did not support chemical control measures. Biological control received more approbation, with 77% of the respondents supporting this approach to a large extent (from mostly to fully support), while only 4% of the respondents did not support this. Given that chemical control measures were unsupported, if we compare it to the other two approaches, data analysis showed that it was statistically significant and that foresters were more likely not to support mechanical control measures (z = -4.549, P = 5.4e-06), while passive NGO members strongly supported it (z = 2.232, P = 0.0256) (Fig. 7).

When asked what measures they would take if the species appeared on their private property (e.g. garden), a vast majority of respondents (81%) would support complete removal. Furthermore, 17% would support partial removal, while only 2% would not


Figure 8. A the extent to which respondents would be willing to remove OLB if it appeared on their private property **B** the extent to which respondents supported the removal of OLB from forests or parks.

support removal of OLB at all (Fig. 8A). When asked what specific method of OLB removal they would use, 49% of respondents would choose a biological method, 31% would choose a mechanical method and only 18% a chemical method.

The removal of OLB as a principal control approach was supported by a large majority of respondents, of these 2% supported insect removal only in forests and 4% in parks, while 91% of them in both settings. Only 2% of respondents did not support removal of OLB if it caused damage (Fig. 8B).

Discussion

The results from the study were encouraging in that the foresters and NGOs were most likely to say that they had seen this discolouration before undertaking the survey, leading us to conclude that they had the basic professional training to distinguish a forest affected by diseases or pests. Furthermore, the fact that 93% of respondents considered this discolouration to be a problem, suggests that there is general public interest and awareness in the health of European oak forests.

The observation that half of the respondents who answered had seen this insect before undertaking the survey, despite its small size and relatively recent introduction into Europe, may be due, on the one hand, to the large numbers of foresters and frequent forest visitors who participated in the questionnaire. However, in part, it is also likely due to the media interest that, in the last few years, has intensely covered the invasions in several cities in Europe where OLB is already established and damage is more visible, such as in Belgrade, Bucharest, Budapest and Zagreb. In addition, the estimation parameters in this study tended to confirm the hypothesis of a previous case study (Japelj et al. 2019) that argued that women or more frequent visitors to the forest, were more likely to correctly recognise an IAS that was not easily visible.

Regarding the occurrence of OLB in the different countries, it was understandable that it is almost impossible to have seen or heard of this insect species before for residents of a country where OLB has only recently been reported or, moreover, if it has not yet been reported. Consequently, these people seem to underestimate the potential consequences of the species. Therefore, programmes to raise public awareness of various invasive species, such as OLB, should be part of a comprehensive future IAS early detection programme.

Although respondents declared having some concern about photosynthesis, forest managers did not consider OLB as a threat to trees and forest health. These results mirror current knowledge on OLB ecology, with severe attacks reducing tree photosynthesis by up to 60% (Nikolić et al. 2019), but given that the radial growth of *Quercus* species mostly occurs in the first part of the vegetation season before OLB symptoms are visible (Szőnyi 1962; Járó and Tátraaljai 1985; Hirka 1991), impacts on tree growth have not been demonstrated so far. However, there is an assumption that the cumulative effect of repeated damage over many years will likely have a significant impact over time (Paulin et al. 2020).

Even though those respondents who were foresters and those associated with environment NGOs were of the opinion that OLB could decrease the value of the wood, it is difficult to quantify how much the value of timber might be influenced by the insects repeated attack until it is established exactly what impact OLB has on radial growth following years of damage.

In terms of property value, private forest owners in our survey were of the opinion that their property would be affected and that, at the same time, people would also avoid entering the forest. To the best of our knowledge, we are not aware of studies exploring the connection between property value and trees infested by OLB. However, another study showed that the invasive species, coqui frog (*Eleutherodactylus coqui*), after it was accidentally introduced into Hawaii, resulted in decreases of up to 64% in property value (Meyerson and Mooney 2007).

The discomfort/annoyance caused by the insect's stings/bites on the human skin is perceived especially by the people who happened to be stung and will become more frequent as people visit infested oak forests and especially those who visit parks in cities where OLB is already present (Paulin et al. 2020). This perception was all the more expected as Europeans have experienced skin inflammation caused by the stings of a similar insect, the sycamore lace bug (*Corythucha ciliata*), in the past (Dutto and Bertero 2013; Izri et al. 2015). In this study, women were more likely to consider that

these stings may be one of the negative effects of OLB on society. Similarly, research conducted in Scotland concluded that women were more likely to be prone to a bad reaction to insect bites (Logan et al. 2010). It is not evident why the parameter estimates suggest that foresters "don't think it has effects", although it could simply be because they consider that OLB has no other effects other than those mentioned by us in the questionnaire.

Our results on the level of support for the removal of OLB complement another study (Japelj et al. 2019) which concluded that respondents were more likely to choose less radical measures for management of invasive plants and animals. In our research, the results showed that this conclusion also applies to invasive insects, more specifically OLB, since our respondents were more likely to choose partial removal of OLB and not its complete eradication. In any case, given the current pattern of invasion across the European oak forests for this species (Mutun et al. 2009; Csepelényi et al. 2017; Simov et al. 2018; Tomescu et al. 2018; Csóka et al. 2019; Paulin et al. 2020), the complete removal would now be impossible. In addition, in this study, women were more reluctant to support complete removal of OLB, which is in line with other studies (Fuller et al. 2016; Japelj et al. 2019). Furthermore, our research showed that the age of respondents also makes a difference. Partial removal was more supported by younger respondents, while complete removal was more supported by older respondents.

The respondents in the survey clearly rejected chemical control measures. This result was in line with both Jetter and Paine (2004) who argue that urban populations prefer to control harmful insects by mechanical or biological methods rather than chemical ones and Japelj et al. (2019) who argue that mechanical and biological control measures are the public's most preferred options for invasive species management. In these two previous studies, respondents chose the mechanical removal method in the first instance and the biological approaches a second option, whilst in our research, respondents preferred biological measures over mechanical ones. In addition to the two previous studies, our research also found women more supportive of biological control measures. Regarding mechanical control measures, there was a difference between foresters and environmental NGOs with the latter stakeholder group finding these measures more acceptable.

Nevertheless, studies to date have shown that mechanical measures are recommended more for early detection, surveying and monitoring and tend not to be used for widespread control programmes. Interestingly, recent management knowledge regarding chemical measures to control OLB seems to suggest that they are also only effective to a certain extent (Bălăcenoiu et al. 2021). For other management approaches, it is not known whether they are likely to work and, to date, no biological agents have been identified that may be influential in reducing OLB populations. Hence, further research into control options is urgently needed, but it is good to know that there is a broad public consent when measures are subsequently developed.

Biological control has strong potential, because the public sees this as a sustainable solution. However, caution should be taken, especially as it can have unwanted side effects as has been seen with *Harmonia axyridis* that was introduced into Europe in 1964

as a biological control agent of aphids on fruit trees (Katsoyannos et al. 1997) or other exotic biological control agents (Van Lenteren et al. 2006). When the assessment of the biocontrol agent is not done properly, the public will be against this option and, more importantly, the released species can become a harmful invasive species itself.

Our results show that our respondents (98%) would support removing OLB if it appeared on their private property, either partially or totally and the results are in line with the study of Japelj et al. (2019) in which 96% of respondents would agree with the elimination of any invasive species if it appeared on their private property. Furthermore, when respondents were asked if they would support removal of OLB from forests or parks if it causes damage, over nine-tenths supported its removal both in parks and in forests.

The strength of our survey was that it was evaluating responses from numerous countries along the invasion gradient. Most comparable studies only cover a smaller geographical area and, therefore, only focus on management of one part of the biological invasion gradient. Taking the whole gradient into account, as done in this current study, enables researchers to see differences in attitudes towards the species in different stages on the invasion of OLB. Of course, cultural and social differences might also play an important factor and it is, therefore, important to focus on these changes in attitudes and use them in communication messages. Our results indicated that there were differences between the different country groups for OLB and, hence, it is important to take this into account for other IAS in the future.

This research is the first pan-European survey which studies the perception of the population on a gradient of invasion caused by OLB. Our study has certain limitations. The study is exploratory, voicing perception, knowledge and attitudes of our respondents and the results cannot be generalised on the entire targeted stakeholder groups in respective countries. However, our results are indicative and, despite not being representative, are still in line with similar studies. Hence, we consider this study to be a good basis for further research on the topic of perception, knowledge and attitudes related to OLB since we can expect that this IAS will certainly spread to other European countries that were not included in this survey.

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Supplementary material I

Annex 1. The questionnaire

Authors: Flavius Bălăcenoiu, Anže Japelj, Iris Bernardinelli, Bastien Castagneyrol, György Csóka, Milka Glavendekić, Gernot Hoch, Boris Hrašovec, Silvija Krajter Ostoić, Marton Paulin, David Williams, Johan Witters, Maarten de Groot Data type: Survey

- Explanation note: The questionnaire used to collect the survey data.
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Supplementary material 2

Appendix 2

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Data type: docx. file

Explanation note: Independent variables descriptions.

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RESEARCH ARTICLE



Non-native plant drives the spatial dynamics of its herbivores: the case of black locust (Robinia pseudoacacia) in Europe

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Abstract

Non-native plants typically benefit from enemy release following their naturalization in non-native habitats. However, over time, herbivorous insects specializing on such plants may invade from the native range and thereby diminish the benefits of enemy release that these plants may experience. In this study, we compare rates of invasion spread across Europe of three North American insect folivores: the Lepidoptera leaf miners *Macrosaccus robiniella* and *Parectopa robiniella*, and the gall midge *Obolodiplosis robiniae*, that specialize on *Robinia pseudoacacia*. This tree species is one of the most widespread non-native trees in Europe. We find that spread rates vary among the three species and that some of this variation can be explained by differences in their life history traits. We also report that geographical variation in spread rates are influenced by distribution of *Robinia pseudoacacia*, human population and temperature, though *Robinia pseudoacacia* occurrence had the greatest influence. The importance of host tree occurrence on invasion speed can be explained by the general importance of hosts on the population growth and spread of invading species.

Keywords

Black locust, Diptera, Lepidoptera, *Macrosaccus robiniella*, *Obolodiplosis robiniae*, *Parectopa robiniella*, *Robinia pseudoacacia*

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Introduction

Plants introduced to new, non-native habitats may have an advantage over the native flora by escaping herbivore pressure, allowing them to allocate more resources toward vegetative and reproductive growth, as formulated e.g. in the enemy release hypothesis (Keane and Crawley 2002). In such a setting, non-native plants can quickly become widespread and invade various habitats. Black locust, *Robinia pseudoacacia* (Fabaceae), is a prime example of this, now being one of the most widespread non-native trees in Europe (Vítková et al. 2016). The native range of this species is limited to the central Appalachian and Ouachita mountains and the Ozark Plateau in the Eastern and Central United States (Huntley 1990). Black locust was introduced to Europe during the first half of the 17th century as an ornamental tree planted in parks and gardens (Wein 1930), and from 1750 on, it was used in forest plantations in Central Europe for purposes of timber and honey production. From these plantings, it spread prolifically and is currently found throughout most of temperate and sub-Mediterranean Europe (Fig. 1A), displacing native vegetation and altering ecosystem properties (Vítková et al. 2016).

Although widely distributed, European populations of black locust were little affected by the few native generalist herbivores feeding on it, with generally marginal impact on the tree (e.g. Bartha et al. 2008). In contrast, five specialist herbivores accidentally introduced to Europe from the native range of black locust were found to have a considerably higher impact on the tree. The first North American insect species discovered feeding on *Robinia* in Europe was the sawfly *Euura tibialis* (Newman, 1837) (Hymenoptera: Tenthredinidae), formerly placed in *Nematus* (Prous et al. 2014). This species was first recorded in Europe in 1825 (Rasplus et al. 2010), twelve years before the species' formal description from the Isle of Wight (Newman 1837). In Europe, this parthenogenetic species is found feeding on *Robinia pseudoacacia* and *R. viscosa*, while in its original North American range it also feeds on *R. hispida* and *Gleditsia triacanthos* (Darling and Smith 1985; Liston 2011).

More recently, four additional *Robinia* herbivores were accidentally introduced from North America to Europe: In 1970, *Parectopa robiniella* Clemens, 1863, a Lepidoptera leaf miner of the Gracillariidae family, was recorded from Northern Italy (Vidano and Marletto 1972). It was followed by *Appendiseta robiniae* (Gillette, 1907) (Aphididae), an aphid first found in 1978 in Italy (Micieli De Biase and Calambuca 1979). Another Gracillariidae leaf miner, *Macrosaccus robiniella* (Clemens, 1859), was first found in 1983 in Northern Switzerland (Whitebread 1990). This species was placed in *Phyllonorycter* Hübner, 1822 until recently, when it was transferred to *Macrosaccus* Davis & De Prins, 2011 (Davis and De Prins 2011). Finally, in 2003 the black locust gall midge, *Obolodiplosis robiniae* (Haldeman, 1847) (Diptera: Cecidomyiidae), was reported from Northeast Italy (Duso and Skuhravá 2002). Upon the arrival of the four most recently introduced *Robinia* herbivores in Europe, black locust was widely distributed and naturalized on the continent. The four herbivore species thus found their food source in abundance and were subject to little competition from more generalist native European herbivores, so that they could extend their distribution range.

Relatively little is known about how the range expansion of specialized non-native herbivorous insects is affected by the distribution of their native host plant in non-native regions. European *Robinia pseudoacacia* and its introduced specialist herbivores are a prime opportunity to study such a setting in more detail. In order to better understand the factors promoting the range expansion of these non-native herbivores and to better predict spread patterns in other parts of black locust's non-native range, we analyze the three most well-documented *Robinia* herbivores present in Europe (*P. robiniella*, *M. robiniella*, and *O. robiniae*), their patterns of historical spread across the continent, and potential factors facilitating this spread. For this, we investigate and quantify different potential drivers of the spread of these herbivores: *Robinia* distribution, human population, mean annual temperature and precipitation, and proximity to previously invaded regions. We hypothesize that both the human population and *R. pseudoacacia* distribution would positively affect herbivore spread via effects on propagule pressure and habitat invasibility.

Methods

In order to avoid confusion among the similar species names, we will refer to the three species by their genus names, i.e., *Parectopa* for *P. robiniella*, *Macrosaccus* for *M. robiniella*, and *Obolodiplosis* for *O. robiniae*. In figures and tables, we state the full species names. We furthermore refer to *Robinia pseudoacacia* simply as *Robinia*, unless other *Robinia* species are mentioned.

Country and regional first records of the presence of *Parectopa*, *Macrosaccus* and *Obolodiplosis* across Europe were obtained from the published literature, online databases and in one case from a photographic record. Coordinates for the localities were obtained through Wikipedia's GeoHack (https://www.mediawiki.org/wiki/GeoHack) and Google Maps (https://www.google.com/maps). Suppl. material 1: Table S1 provides a full list of records for the three folivore species. We also obtained georeferenced occurrence records for each of these three species at a global scale. These were sourced from GBIF (https://www.gbif.org), EPPO (https://gd.eppo.int), CABI (https://www. cabi.org/ISC), Davis and De Prins (2011) and Shang et al. (2015). These global records were not used for analysis of spread rates.

Radial rates of spread were estimated for each species from European first records using the distance regression method (Gilbert and Liebhold 2010). According to this method, a linear regression model was fit to the distance from the first discovery point in Europe as a function of year of first discovery. The slope of the estimated regression equation provides an estimate of the radial rate of range expansion. Distances between the distribution records were calculated with the R packages geosphere 1.5-10 (Hijmans et al. 2019) and sp 1.4-2 (Pebesma and Bivand 2005; Bivand et al. 2013), using the 'Vincenty' (ellipsoid) great circle distance function (distVincentyEllipsoid). Linear regressions were performed using the *lm* function in the R language.

In order to explore factors affecting spread of each species, we applied Cox Proportional Hazard analysis following the approach used by Ward et al. (2020). This model quantifies the probability that each uninvaded location will become invaded at annual time steps as a function of a series of candidate explanatory variables. Five predictors for herbivore spread were considered: human population, Robinia distribution, mean annual precipitation, mean annual temperature (see Fig. 1), and spatial proximity. Human population (expressed as number of inhabitants in the year 2000) was extracted from a human population density raster at a resolution of 30 arc-seconds from the Global Rural-Urban Mapping Project (Balk et al. 2006). Data on Robinia distribution (expressed as total tree area in km²) were extracted from the European Atlas of Forest Tree Species (Sitzia et al. 2016) as a relative probability of presence raster at a resolution of 1 km, based on the C-SMFA model and field observations (de Rigo et al. 2016). Values of total annual precipitation (cm) and annual mean temperature (°C) for the period 1970-2000 were obtained from the WorldClim v2 database (Fick and Hijmans 2017) at a resolution of 30 arc-seconds. No data on Robinia distribution were available for points located in Moldova, Belarus, Ukraine and the European part of Russia. Values for each variable were calculated for areas in a 10 or 50 km buffer radius zones around each of the individual records for each species. Spatial proximity (sp) to previously invaded points (associated with diffusive propagule pressure) was a time-varying predictor and calculated as:

spatial proximity
$$sp = \sum_{i=1}^{n} \frac{1}{d_{ij}}$$
,

where d is the distance (in km) between a given point i and each previously invaded point j. Thus, spatial proximity was estimated for each point in each year, while all other predictors did not change annually. Human population and *Robinia* distribution were log-transformed to reduce skewness.

In addition to locations of individual records for each species, the Cox proportional hazard model was fit using "pseudo-absence" points. These are locations falling outside of the invaded range of each species that were never invaded during the time span of records. Pseudo-absence records were generated in a 50 km grid across a 300 km buffer zone outside of the minimum convex hulls around each set of records for each species (see Fig. 2). The minimum convex hull, individual buffer zones and spatial statistics for the selected variables were created using ESRI ArcMap 10.5.1 (ESRI 2016).

Given uncertainty about the identity of most relevant spatial scales of the predictor variables, all possible combinations of 10 km and 50 km scale predictors were fit in full models. The model with the lowest Akaike Information Criterion (AIC; Akaike 1973) was then further reduced (if applicable) by iteratively removing predictors with the higher *p*-value until all remaining were p < 0.05. To assess robustness of our models to the missing values of *Robinia* density for Eastern Europe, the entire model fitting and selection process was redone without considering *Robinia* distribution as a predictor. Models were fit using the R package survival 3.2-7 (Therneau 2020).



Figure 1. Variables investigated for their influence on the spread of the three *Robinia*-specific herbivores in Europe **A** estimated distribution of *Robinia pseudoacacia* **B** human population **C** mean annual precipitation **D** mean annual temperature.

Results

We assembled 97 first record locations from 24 countries for *Parectopa*, 92 locations from 25 countries for *Macrosaccus*, and 75 locations from 33 countries for *Obolodiplosis* (Fig. 2; Suppl. material 1: Table S1; Mally et al. 2021). Linear regressions show a highly significant correlation between time and distance from the invasion focus for all three herbivores: the radial rate of spread estimated by linear regression (Fig. 3) is 35.4 ± 5.7 km/ year ($t_{95} = 6.16$, p < 0.005) for *Parectopa*, 73.42 ± 5.0 km/year ($t_{90} = 14.79$, p < 0.005) for *Macrosaccus*, and 128.3 \pm 8.1 km/year ($t_{73} = 15.79$, p < 0.005) for *Obolodiplosis*.

Macrosaccus mainly spread east- and northward in the first two decades after its introduction (Fig. 2B), as did *Parectopa*. The latter species was first discovered in Northern Italy, south of the Alps. In order to reach the areas north of the Alps, it spread east- and later northward around this mountain range that acted as a geographical barrier (Fig. 2A). *Obolodiplosis* spread more or less equally in all directions from its first occurrence location in Northern Italy. Within Europe, it is the most widespread of the three investigated *Robinia* herbivores, with distribution records stretching from Portugal to the Caspian Sea and from Sicily to Southern Sweden and the Baltic states



Figure 2. European records for **A** *Parectopa robiniella* **B** *Macrosaccus robiniella*, and **C** *Obolodiplosis robiniae*. The first European record for each species is marked by a star, the subsequent spread is indicated by color-coded records in 5-year (**A**, **B**) or 2-year (**C**) intervals. The grid of black points around the distribution areas marks pseudo-absence locations in a 300 km buffer region formed by the minimum convex hull around the records for each species.

(Fig. 2C). In the 18 years since its first discovery in Europe, it has invaded a larger area than either of the two leaf miners, which had been introduced considerably earlier.

Results of the reduced Cox proportional hazard models are shown in Table 2, correlation matrices of predictors for the best-fitting model for the three species in Suppl. material 2: Tables S4–S6, and Akaike Information Criterion (AIC) values for the three species in Suppl. material 2: Tables S7–S9. Annual mean precipitation is found to have the least predictive power among the five investigated predictors. It is absent in all reduced models (Table 2), and is significant only for *Parectopa* in the full model (Suppl. material 2: Table S2). In the reduced (Table 2) and full (Suppl. material 2: Table S2) models, colder annual mean temperatures were associated with an increased risk of invasion for *Parectopa* and *Macrosaccus* (as indicated by the negative Z-scores), and less so for *Obolodiplosis*. In the models with *Robinia* omitted (Suppl. material 2: Table S3), it is significant for *Parectopa*, and less so for *Macrosaccus*; no significance is observed for *Obolodiplosis*. In the full and the reduced models, human population has a highly significant positive influence on the invasion risk for *Parectopa* and *Macrosaccus*, and less so for *Obolodiplosis*. In the models with *Robinia* omitted (Suppl. material 2: Table S3), it is highly significant for all three species. *Robinia* distribution is found to be the most



Figure 3. Linear regression scatterplots of distance (in km) from first record in Europe over time, for **A** *Parectopa robiniella* **B** *Macrosaccus robiniella*, and **C** *Obolodiplosis robiniae*.

Table 1. Results of the linear regression of distance over time for the three herbivore species. Radial rate of spread (km per year) is provided by the slope of the regression.

| | Intercept \pm SE | Slope (radial rate of spread) \pm SE | Multiple R-squared |
|------------------------|----------------------|--|--------------------|
| Parectopa robiniella | -10.06 ± 201.60 | 35.37 ± 5.7 | 0.29 |
| Macrosaccus robiniella | -354.72 ± 107.55 | 73.42 ± 5.0 | 0.71 |
| Obolodiplosis robiniae | 270.69 ± 84.08 | 128.29 ± 8.12 | 0.77 |

Table 2. Results of reduced Cox proportional hazards (CPH) models with lowest AIC and all predictors with p < 0.05.

| Species | Predictor | Coefficient | SE | Z | p |
|------------------------|--------------------------|-------------|--------|-------|----------|
| Parectopa robiniella | spatial proximity sp | 3.67 | 0.80 | 4.59 | < 0.0001 |
| | human population (50 km) | 0.61 | 0.09 | 6.61 | < 0.0001 |
| | Robinia (10 km) | 0.59 | 0.07 | 7.89 | < 0.0001 |
| | temperature (50 km) | -0.61 | 0.08 | -8.10 | < 0.0001 |
| | precipitation (10 km) | -0.0032 | 0.0011 | -2.98 | 0.0029 |
| Macrosaccus robiniella | spatial proximity sp | 22.87 | 2.61 | 8.78 | < 0.0001 |
| | human population (10 km) | 0.58 | 0.08 | 7.64 | < 0.0001 |
| | Robinia (50 km) | 0.40 | 0.06 | 6.76 | < 0.0001 |
| | temperature (50 km) | -0.58 | 0.09 | -6.37 | < 0.0001 |
| Obolodiplosis robiniae | spatial proximity sp | 40.08 | 15.74 | 2.55 | 0.0109 |
| | human population (10 km) | 0.37 | 0.11 | 3.35 | 0.0008 |
| | Robinia (50 km) | 0.44 | 0.06 | 7.05 | < 0.0001 |
| | temperature (50 km) | -0.13 | 0.06 | -2.07 | 0.0382 |



Figure 4. Global distribution of **A** *Robinia pseudoacacia* **B** *Parectopa robiniella* **C** *Macrosaccus robiniella* and **D** *Obolodiplosis robiniae* compiled from our own dataset, GBIF, EPPO, CABI, Davis and De Prins (2011) and Shang et al. (2015).

consistent predictor, explaining the spread of all three species with high significance both in the full and the reduced models. In the full and the reduced models, proximity to previously invaded areas is highly significant for *Parectopa* and *Macrosaccus*, but much less so for *Obolodiplosis*. In the models without *Robinia*, it is highly significant for all three species, along with human population.

The known global distribution of *Robinia* is shown in Fig. 4A, and the distributions of the three herbivore species are shown in Fig. 4B–D. *Robinia* is widely distributed in virtually every temperate and subtropical portion of the world. The distributions of the three herbivore species appear to be more limited. Of the three species, *Obolodiplosis* is the most widely distributed, having established in Europe, East Asia and New Zealand. However, there is no record of its presence in either the Afrotropic or Neotropic regions. The two Lepidoptera species *Parectopa* and *Macrosaccus* appear to be slightly less successful invaders, having only established in Europe.

Discussion

The three herbivores show similar patterns of radial range expansion in Europe, although with substantially different annual spread rates. All three species were initially discovered in the same general region of south-central Europe with only ~200–400 km separating their sites of initial discovery. Strikingly, *Parectopa*, which was the first of the three investigated *Robinia* herbivores to be recorded from Europe over 50 years ago, has the smallest annual spread rate (about 35 km/year) and is reported from the fewest number of countries (24). *Macrosaccus*, first reported 13 years later in 1983, exhibits an average spread rate of 73 km/year, but spread much faster in Hungary with its abundant black locust stands, invading the entire country from west to east in two years (Csóka 2001). The species is currently recorded from 25 European countries. The newest invader, *Obolodiplosis*, has the by far highest spread rate (128 km/year) and has spread to 33 countries since its first report in 2003. Of the three species, *Obolodiplosis* has also spread the most widely on the global scale (Fig. 4D). While *Obolodiplosis* has successfully invaded Europe, East Asia and New Zealand, *Parectopa* and *Macrosaccus* have only invaded Europe. In North America, all three species have also extended their range beyond the native range of *Robinia*, with *Macrosaccus* and *Obolodiplosis* having spread as far as the west coast of the US and Canada.

Invasion spread is driven by population growth coupled with movement. Thus, any factors that affect either population growth or movement are likely to influence patterns of spread. It is likely that the differences in invasion patterns observed among these species (both within Europe and globally) can be attributed to their biological traits that influence their population growth rates or dispersal, either natural dispersal or accidental long-distance movement by humans. Obolodiplosis develops through three generations per year in the Czech Republic, and in up to four generations in more southern regions such as Italy, Hungary and Serbia (Skuhravá et al. 2007; Mihajlović et al. 2008; Duso et al. 2011). For China, however, up to six generations per year have been reported (Shang et al. 2015). The capacity for this species to develop through multiple generations likely facilitates rapid population growth (Fahrner and Aukema 2018). The small size of adults also probably leads to this species being easily transported in wind though such natural dispersal probably only facilitates local dispersal. Long-distance transport (including inter-continental spread) is most likely to occur via hitch-hiking with cargo, vehicles, etc. Pupation of *Obolodiplosis* takes place in the galls, except for the last generation of a year, where pupation takes place in the soil (Uechi et al. 2005; Tóth et al. 2009). Because this species overwinters as a diapausing larva (Duso et al. 2011), this probably creates potential for the species to be accidentally transported long distances with vehicles and other objects that might be placed under *Robinia* trees prior to transport.

Even though both of the two leaf miner species belong to the same Lepidoptera family (Gracillariidae), their biologies exhibit differences that potentially explain differences observed in their success and rate of spreading across Europe. *Parectopa* produces two to three generations per year, with two in more northern regions such as Belarus, and up to three in more southern regions like Transnistria (Moldova) and Croatia (Maceljski and Igrc 1984; Antyukhova 2010; Sautkin and Evdoshenko 2012). *Macrosaccus* is reported to produce two to five generations per year: two generations in Southern Germany, Switzerland and Austria (Wipking 1991; Huemer et al. 1992; Huemer 1993; Rietschel 1996), two to three generations in Hungary (Csóka 2001), three generations in Serbia and Belarus (Stojanović and Marković 2005; Sautkin and Evdoshenko 2012), four generations in Slovenia (Seljak 1995), and potentially even a fifth generation in Croatia (Maceljski and Mešić 2001). Furthermore, often two to three (and up to eight) *Macrosaccus* larvae share a common mine (Huemer 1993; Rietschel 1996; Šefrová 2001), whereas *Parectopa* caterpillars usually inhabit

mines solitarily (Baugnée 2014). In addition to a generally higher reproduction rate, *Macrosaccus* may thus be able to attain a higher population density.

Pupation takes place in the leaf litter in the case of *Parectopa*, whereas *Macrosaccus* larvae pupate on the leaves (Antyukhova 2010; Davis and De Prins 2011). In urban areas, *Parectopa* pupae might therefore be removed with the leaf litter in the autumn (Antyukhova 2010), whereas *Macrosaccus*, which overwinters in the adult stage (Deschka 1995), probably remains on or near its host plants, increasing its chances of reoccupying *Robinia* stands in the following season. However, pupating in the leaf litter, where it is presumably less exposed to parasitoids, might increase the survival of *Parectopa* as compared to *Macrosaccus* (Csóka et al. 2009). Given that *Parectopa* exhibits the slowest rate of spread of all three species, we can hypothesize that their biology of overwintering as pupae in leaf litter does not facilitate their anthropogenic movement to the extent seen in *Macrosaccus* and *Obolodiplosis*.

The small adult body size and wing anatomy of the two leaf miners indicate that they likely spread passively with wind, but transport of hibernating or resting adults with trade cannot be excluded (Rietschel 1996; Šefrová 2001, 2003). Passive wind transportation might explain the generally stronger eastward spread of the leaf miners with the prevailing west winds in Central Europe.

We find a negative correlation between mean annual temperature and the spread of the two leaf miners, meaning that colder temperatures promote the spread of these species. Considering the geographical setting in which the range expansion of these species occurred, this is not surprising: with their first records in Northern Italy resp. Northern Switzerland, range expansion would occur mostly north- and eastward, as expansion southwards is limited by the Mediterranean Sea. The negative correlation between temperature and spread might thus be a result of generally more sampling points in the north- and eastward direction of the points of first record, where annual mean temperatures are generally lower than those in Northern Italy (see Fig. 1D).

Our findings of colder annual mean temperatures promoting the spread of both leaf miners are in contrast to published information at least of *Parectopa*, which is reported to be "more thermophilous" than *Macrosaccus* (Baugnée 2014). This is consistent with its slower northward spread and its presence in Southern Italy (i.e., south of the Emilia Romagna region), where *Macrosaccus* is absent (Stoch 2003). *Parectopa* was also reported as "massively present" with 50–80% of leaflets infested in the hot and dry, sandy environments of coastal Croatia, whereas habitats in inland Croatia with a more continental climate experienced a low infestation rate of 3% (Maceljski and Igrc 1984; Stojanović and Marković 2005). *Parectopa* might therefore continue its spread in the more southern parts of Europe and into the Transcaucasian region where its hostplant is present. Fodor and Hâruța (2009) find almost no niche overlap between *Parectopa* and *Macrosaccus* in Romania, despite both utilizing *Robinia* leaves as their food source, where they occupy mostly opposite sides and different parts of the leaflets. The two leaf miners are thus not in direct competition for their food source.

Both leaf miner species are often reported to exhibit high population densities during their initial colonization phase following establishment in a new region, while subsequently becoming much rarer (Seljak 1995; Šefrová 2001; Tomov 2003; Antyukhova 2010; Baugnée 2014). In Poland however, Parectopa was mostly first recorded from single mines in isolated locations, apparently as a result of anemochorous dispersal. The following absence of *Parectopa* mines in these locations for several years suggests that these founder populations were unable to establish. More successful northward spread of Parectopa occurred along river valleys, e.g. the Vistula valley, where Robinia finds favorable growing conditions on the sunny slopes (JB, pers. obs.). Macrosaccus, on the other hand, quickly spread through Poland over a wide front and in considerable abundance until 2005, when areas of rarer Robinia occurrence (presumably due to less suitable growth conditions) were reached (JB, pers. obs.). There are also records of Obolodiplosis being very abundant in recently invaded regions, particularly in East Asia (Yang et al. 2006). Though lacking quantitative data, it appears that none of the three species is particularly abundant in their native range in North America (AML, pers. obs.). Along these lines, we note that most of the records of *Obolodiplosis* from North America lie outside of the native range of its host, Robinia, which may be indicative of the low abundance of *Obolodiplosis* in its native range.

Parasitization might play an important role in the speed of spread. Since their establishment in Europe, the two leaf miners have accumulated a large number of generalist parasitoids (summarized in Serini 1990; De Prins and De Prins 2006–2020, and Csóka et al. 2009), with 20 species recorded for Parectopa, and 37 for Macrosaccus. Parasitization rates vary considerably though, ranging in the case of Macrosaccus from 1–3% in Upper Austria (Deschka 1995), 10–30% in Southern Moravia (Šefrová 2001), <40% in Kraków, Poland (Wojciechowicz-Żytko and Jankowska 2004), 35-50% in Trentino, Italy (Angeli et al. 1996), up to 47.6% in Hungary (Csóka et al. 2009), and >60% in Bosnia-Herzegovina (Dimić et al. 2000), to 30-67.5% in Serbia (Stojanović and Marković 2005). Information on parasitization rates in Parectopa are few, reaching a maximum of 15.3% in Hungary, where *Macrosaccus* is up to three times more heavily parasitized (Csóka et al. 2009). Obolodiplosis hosts few parasitoids, which likely promotes its rapid spread in Europe and other regions of the world. It is to be expected that Obolodiplosis will have a fairly large impact on Robinia populations wherever it is introduced, which might however be compensated by the fast growth and reproductive abilities of Robinia. On the other hand, Platygaster robiniae, the gall midge's primary parasitoid infesting the host eggs and feeding gregariously on the larvae (Buhl and Duso 2008; Duso et al. 2011; Kim et al. 2011), is reported to cause parasitization rates of 51.6% to 84.8% (Park et al. 2009; Lu et al. 2010), making it a promising candidate as control agent of the locust gall midge (Lu et al. 2010).

Our quantitative analysis indicates local *Robinia* density to be the single factor having the strongest impact on the spread of *Parectopa*, *Macrosaccus* and *Obolodiplosis* across Europe. Skuhravá et al. (2007) reached a similar conclusion for *Obolodiplosis* based on a qualitative evaluation of historical European spread. Since feeding of all three insect species is limited to *Robinia*, it is understandable that its density would strongly affect population growth rates and consequently affect spread. Several other studies have reported that host densities influence rates of invasion spread of invading species (e.g., Meier et al. 2014; Hudgins et al. 2017; Ward et al. 2020). The resource concentration hypothesis posits that more abundant host plant resources promote insect herbivore population growth rates (Hambäck and Englund 2005), and such elevated rates can be expected to translate into increased invasion spread rates.

The fact that *Robinia* is itself an invasive species has interesting implications regarding the positive effect of *Robinia* density on spread of these folivore species. It has been noted that at a global scale, plant invasions or widespread planting of non-native plants promote invasions by herbivore species that use these plants as hosts (Liebhold et al. 2018; Branco et al. 2019; Guo et al. 2019). There are many examples in which abundant distributions of non-native plants have promoted invasions by insect herbivores that specialize on those plants (e.g., Hurley et al. 2016). This phenomenon can be regarded as a type of "invasion meltdown" where invasion by one species triggers subsequent invasions of other species (Simberloff and Von Holle 1999). However, less is known about how host insect invasions can mediate invasions of their parasites beyond theoretical studies (e.g., Fagan et al. 2002).

Previous studies have also identified human population density to be related to the spread of invading insect species (Gilbert et al. 2004; Ward et al. 2020). It is logical that humans may accidentally transport insect life stages and therefore promote long-distance dispersal. Population models show that when occasional long-distance dispersal is coupled with frequent short-distance dispersal, this leads to much greater rates of spread than when long-distance spread is lacking (Shigesada et al. 1995; Hastings et al. 2005). Long-distance dispersal is often associated with passive movement by humans and thus high human densities may drive higher rates of longdistance movement and thereby facilitate invasion spread (Gippet et al. 2019). But the significant influence of human population may also be confounded with *Robinia* occurrence since human-caused disturbance typically promotes this tree species (Vítková et al. 2016).

Similar to human population, annual mean temperature was found to have a significant influence on the spread of the two leaf miners, but less so for the gall midge. This result is in concordance with the wider climate spectrum of invaded regions of *Obolodiplosis*: in Europe, the gall midge is now distributed from the hot-summer Mediterranean climate of Portugal, Sicily and Greece to the humid continental climate of Southern Sweden and the Baltic states. On the global scale, it has been recorded from Vancouver Island, Canada (Skuhravá et al. 2007), Japan and South Korea (Kodoi et al. 2003; Woo et al. 2003; Uechi et al. 2005; Tokuda et al. 2019), China (Yang et al. 2006; Shang et al. 2015), the Russian Far East (Csóka et al. 2017), and New Zealand (Anonymous 2009; Bain 2009) (Fig. 4D). *Parectopa* and *Macrosaccus*, on the other hand, have only been reported outside their native range from Europe and the west coast of North America (Fig. 4B, C). In contrast to mean temperature, we found annual mean precipitation to have no significant influence on the spread of the three herbivore species.

Our results indicate that spatial proximity to previously invaded regions plays an important role for the spread of *Parectopa* and *Macrosaccus*, but much less so for the gall midge Obolodiplosis. Obolodiplosis showed an extremely fast spread across most of Europe in the 18 years since its first record in Europe, now occupying a considerably larger area than the much earlier established leaf miners. The results of Roques et al. (2016) indicate that it spread faster than any other insect species invading Europe in their analysis. The spread of this species exhibited several longdistance jumps to form discontinuous populations, the most prominent one being a 2,000 km dispersal from its first record in Northeast Italy to the East Ukraine in just three years. Furthermore, Obolodiplosis successfully invaded the islands of Great Britain, Sicily, Corfu, and the Balearic Islands, none of which have been reached by either of the two leaf miners. Skuhravá et al. (2007) speculate that the gall midge may frequently be transported over long distances with nursery trees, and/or through passive transport by freight traffic. Our finding that spatial proximity to previously invaded areas plays a minor role in the spread of Obolodiplosis concord with its high spread rate of 128.3 km/year, and the observed long-distance dispersal.

None of the scatterplots of the three herbivore species (Fig. 3) show a clear establishment phase preceding the expansion phase. Macrosaccus, however, was closely monitored in the area of its first discovery in 1983 around Basel in Northern Switzerland. There, the distribution range did not exceed an 85 km radius around Basel by 1989, although the species was "already common around Basle in 1983" (Whitebread 1990). The absence of a clear establishment phase in the scatterplots might indicate that the species arrived some years before their first record in Europe, when they had time to establish a sufficiently large population and propagule pressure to expand their ranges. The scatterplots also provide little evidence of geographical "saturation" in any of these three species. As invading species spread to all suitable areas in a region, such plots can be expected to asymptotically stop increasing (Shigesada and Kawasaki 1997). Eventually, all three species can be anticipated to become established in all regions with suitable habitat. That environmental niche is presumably defined both by the presence of a suitable climate and by the presence of Robinia hosts. Comparison of the current distribution of these species (Fig. 2) with the distribution of Robinia suggests that all three species will soon saturate their potential habitat. However, on a global scale these same species are far from saturation and further invasions can be anticipated in the future.

In addition to these three species that utilize *Robinia* as a host, Hargrove (1986) identified 72 other herbivore species associated with *Robinia* in its native North American range. Given the three species studied here, along with *Euura tibialis* and *Appendiseta robiniae*, it is evident that only five out of 75 North American *Robinia* specialists have presently invaded Europe. Thus, we can anticipate that additional herbivore species are likely to invade Europe and elsewhere in *Robinia*'s invaded range and that this will contribute to the dilution of enemy release in populations of this invasive plant.

Conclusion

Specialist herbivores are crucially dependent on the presence of their host plant. Our results show that the widespread presence of *Robinia* in Europe, especially in humaninfluenced environments, greatly facilitated the spread of the introduced North American herbivores. The excessive proliferation of *Robinia* increases the likelihood of establishment and spread of non-native specialist herbivores, thus creating a negative feedback where the initial beneficial effects of enemy release on *Robinia* are diminished, and *Robinia* populations are potentially reduced.

With *Robinia* having been introduced to most regions of the world with a suitable temperate climate, conditions are thus beneficial for the establishment of these insects, and potentially other specialist herbivores from black locust's native range. *Obolodiplosis* has already become established in East Asia and New Zealand, where it has exhibited rapid spread similar to that in Europe. Its success can be attributed to the ability for long-distance jumps as well as to life history traits, such as high reproduction rates, and a presumably small guild of parasitoids. For the two leaf miner species, spatial proximity to previously invaded areas is another important factor affecting range expansion, reflecting the ability of these species to disperse into adjacent uninvaded areas following initial colonization. Although the three investigated herbivores invaded Europe under similar conditions, there are pronounced differences in their invasion success, which can be explained with species-specific life history traits. Furthermore, pan-European cargo traffic has increased over the past decades, increasing the likelihood of long-distance spreading.

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Supplementary material I

Table S1. First record locations of *Parectopa robiniella*, *Macrosaccus robiniella* and *Obolodiplosis robiniae* from Europe.

Authors: Richard Mally

Data type: occurrences

- Explanation note: An XLSX worksheet containing three tabs, one for each of the three investigated black locust herbivores *Parectopa robiniella*, *Macrosaccus robiniella* and *Obolodiplosis robiniae*, with the first locations (with country, administrative area, city and specific locality, where available), longitude, latitude, observation year and reference of the record.
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Supplementary material 2

Tables S2–S9

Authors: Richard Mally, Samuel F. Ward, Jiří Trombik, Jaroslaw Buszko, Vladimir Medzihorsky, Andrew M. Liebhold

Data type: docx. file

- Explanation note: Table S2. Results of full Cox proportional hazards (CPH) models for all predictors with *p* < 0.05. Table S3. Results of full Cox proportional hazards (CPH) models with *Robinia* distribution removed as predictor, with *p* < 0.05. Table S4. Correlation matrix of predictors for best-fitting model for *Parectopa robiniella*. Table S5. Correlation matrix of predictors for best-fitting model for *Macrosaccus robiniella*. Table S6. Correlation matrix of predictors for best-fitting model for *Macrosaccus robiniella*. Table S7. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S8. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*. Table S9. Akaike Information Criterion (AIC) values for *Macrosaccus robiniella*.
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RESEARCH ARTICLE



Invasive hornets on the road: motorway-driven dispersal must be considered in management plans of Vespa velutina

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Abstract

Understanding the mechanisms that potentiate the dispersion of an invasive species is essential to anticipate its arrival into new regions and to develop adequate management actions to minimize damage to biodiversity and society. One of the most successful invaders in Europe, the yellow-legged hornet (*Vespa velutina*), is dispersing through self-diffusion and jump dispersal. Using information on species occurrence in Portugal from 2013 to 2018, this study aimed to understand the range expansion trajectory of *V. velutina* and to identify the role of climate, landscape and anthropogenic variables on the two mechanisms of spread. We found that in Portugal the invasion is proceeding faster southwards (45 km/year) along the Atlantic coast than eastwards (20 km/ year) where the climatic suitability gradient is more compressed, with jump dispersal playing an important role in this difference and in the acceleration of the invasion process. Dispersal by diffusion was best explained by the annual range of temperature and precipitation of the wettest month, with distance to shrub land also having an important role. Additionally, jump dispersal appeared to be facilitated by motorways, hinting at the role of human-mediated dispersal. Indeed, the number of nests that resulted from this dispersive mechanism were significantly closer to motorways than expected by chance. To prevent the dispersal of *V. velutina* into Mediterranean regions, and in addition to a special attention to the advancing front, early monitoring programs should also target a buffer zone on both sides of motorways, and at freight shipping hubs.

Keywords

Climatic gradient, diffusion dispersal, human-mediated dispersal, jump dispersal, motorways, Vespa velutina

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Introduction

Invasive species can have important environmental and socioeconomic impacts. Knowing the dispersal routes of such species is crucial to anticipate their arrival and define adequate management practices in a timely fashion. Invasiveness (a dynamic property of the species) and invasibility (a property of a location that can change with anthropogenic disturbance, seasons, climate change), two key components of biological invasions, are thought to be primarily determined by species' dispersal ability and habitat suitability, respectively (Brooks 2007). Invasiveness is thus mediated by life strategies (Pysek and Richardson 2007), whereas invasibility is related to local conditions at the site, habitat or landscape levels (Vicente et al. 2010). For a risk assessment of the vulnerability of a site to an invasive species it is therefore important to have detailed information on both the species' ecological niche (i.e., static information, such as climatic tolerance) and movement ability, which will regulate if and when suitable areas away from the site where the invasion started will be reached (i.e., dynamic information). Including such information on distribution models of invasive species can help to distinguish suitable habitat that is, or can be potentially occupied, from suitable habitat that is inaccessible (Miller and Holloway 2015).

As different environmental conditions and landscape heterogeneity may accelerate or hamper the invasive process (Hastings et al. 2005), it is also important to identify the patterns of range expansion. Three different trajectories of range expansion versus time can be considered: linear (Andow et al. 1990), biphasic (with an initial shallow slope followed by a steep linear slope), and accelerating with time, quickly reaching the saturation phase (Shigesada et al. 1995). The three expansion patterns occur through either self-mediated dispersal from an initial location (diffusion; Lockwood et al. 2007) or jump dispersal into regions relatively far from the core distribution area without colonizing the regions in between, leading to the establishment of nascent colonies (Suarez et al. 2001), "outposts" hereafter. If outposts establish in environmentally suitable areas the species can continue the expansion process from there, accelerating it. When a species spreads through both processes (natural diffusion and jump dispersal), stratified diffusion occurs (Hengeveld 1989; Suarez et al. 2001). In such a process, the initial range expansion occurs mainly by diffusion, but as the area of the founder population expands, new nests created by long-distance events accelerate range expansion in later phases (Shigesada et al. 1995; see also some insect examples in Andow et al. (1990)).

Insects are the dominant group among non-native terrestrial invertebrates in Europe (Roques et al. 2009). Social insects, in particular social Hymenoptera, are likely to become successful invaders due to their excellent dispersal abilities, high reproductive rates in an annual life cycle, broad diets and habitat ranges, colony initiation by a single inseminated queen, and to their close association with human transportation with relatively low probability of detection (Moller 1996; Beggs et al. 2011). These characteristics favor the invasiveness of eusocial insects, giving them a plasticity of responses that allow their survival and establishment in new environments. Impacts of invasive social insects include changes of ecosystem functions, competitive displacement of

native species, hybridization with native species, threats to human or animal health through stings or the transmission of pathogens (Schneider et al. 2004; Lester and Beggs 2019). One example of an invasive eusocial insect that is currently spreading in Europe is the yellow-legged hornet, (Vespa velutina Lepeletier, 1836), an aggressive predator of honeybees and wild pollinators, that is putting honey and agricultural production at risk (Monceau et al. 2014; Verdasca et al. 2021). The high dispersal ability of this invasive combined with the lack of true competitors and with the availability of food resources in Europe, has been favoring its expansion in this continent. After the accidental introduction of V. velutina in France in 2004, probably by a single female originating from temperate south-eastern China (Arca et al. 2015), the invasion spread to other European countries - Spain, Portugal, Belgium, Italy, Germany, United Kingdom, Netherlands and Luxembourg, being the hornet now considered an invasive alien species of concern in the European Union (European Commission 2016). In 2011, a new invasion was detected in the north of Portugal (Grosso-Silva and Maia 2012) that then expanded southward into the center of Portugal (Carvalho et al. 2020) across a climate gradient between Temperate and Mediterranean bioclimates (Rivas-Martínez et al. 2017; Sayre et al. 2020) and northward into Galicia (Spain) (Rodríguez-Flores et al. 2019). The current European distribution of this species, mostly concentrated along the Atlantic coast, aligns with the climatic preferences predicted by Villemant et al. (2011). As a central place forager, workers of V. velutina optimally explore foraging areas 500 m to 800 m from their nests, although the maximum homing ability (the maximal distance an individual is able to travel on its way home) of the hornet is much greater (5000 m; Poidatz et al. 2018).

The spread of V. velutina in Europe has been considered a stratified diffusion process, including a mixture of natural diffusion and jump dispersal events (Bertolino et al. 2016; Robinet et al. 2017; Lioy et al. 2019). Jump dispersal may occur by two different processes: self-dispersal of gynes (queens of the next generation), which are able to fly long distances on their own (18 km/day in flight mill experiments - pers. comm., Dr. D. Sauvard, INRA, France to the authors of Robinet et al. 2017), or through human-mediated dispersal (Robinet et al. 2019). Due to the difficulty in disentangling these processes, only occurrence points found more than 78 km (yearly spread rate in France) from their nearest neighbors were considered to result from long distance dispersal events by Robinet et al. (2017). However, Bertolino et al. (2016), working in Northwest Italy, considered a much shorter distance as the limit over which human-mediated dispersal was considered the most likely explanation for jump dispersal events (yearly spread rate in Italy = 18.3 ± 3.3 km). Besides the different methodological approaches (including the scale of analysis), the differences between the two studies are attributed by Bertolino et al. (2016) to the Italian mountainous territory, when compared with the largely flat north western and central France, while Robinet et. al. (2017) justify the high spread rate in France by the ability of founder females of flying long distances in flight mill experiments.

Precipitation and temperature are thought to be the strongest predictors of the invasive range of *V. velutina* (Villemant et al. 2011), with land-use also playing an

important role at a regional scale (Bessa et al. 2016). This hornet is now colonizing the Mediterranean peninsulas, and therefore being exposed for the first time to a Mediterranean climate. As the species spreads along the transitional temperate-Mediterranean climate regions, it is important to assess the extent of suitable area at a finer resolution, as well as to understand how the invasion process is unfolding and the role of humanmediated dispersal. A recent study highlighted the need to identify the colonization pathways and plan management approaches to halt the spread of *V. velutina* in Portugal (Carvalho et al. 2020). At a regional scale, the identification of a limited number of key variables explaining the dispersal and establishment success of *V. velutina* can facilitate the creation of effective preventive and control measures. In this study, we incorporated land cover and anthropogenic drivers to predict the risk of invasion by *V. velutina* with-in the transitional temperate-Mediterranean zones and infer colonization pathways. More specifically, we (i) assessed the roles of diffusion and jump dispersal on *V. velutina* expansion into Mediterranean-type climates and (ii) identified which environmental attributes are most influential on the direction and speed of its dispersal.

Material and methods

Nest occurrence data in Portugal

For this study, we focused on the secondary introduction event of the hornet in Portugal and used all of the available Portuguese presence data of V. velutina (8610 records of nests, from 2013 to 2018). Data was obtained from Bombeiros Voluntários de Viana do Castelo and from the online platform 'STOPvespa' (http://stopvespa.icnf.pt/), which is managed by the Instituto da Conservação da Natureza e das Florestas (ICNF) and aggregates all validated Portuguese records of V. velutina nests that were previously registered in the platform by citizens. To avoid spatial autocorrelation, we reduced the number of occurrence data points through the spatially rarefy occurrence data tool (pixel size resolution: 300 m) in SDMtoolbox (Brown 2014) in ArcGIS 10.4.1 (ESRI 2016); this resulted in a total of 7847 points (Suppl. material 2: Appendix A). To calculate the continuous distribution area of V. velutina (2013 to 2018) we drew a 5 km buffer around each nest. The workers of V. velutina probably forage less than 1000 m from their nest, and this buffer of 5 km corresponds to the estimated maximum homing ability of the species, since few workers have the ability to perform long trips (Poidatz et al. 2018). Moreover, and according to (Lioy et al. 2019), most new nests (>90%) in NW Italy were located within this radius to their nearest source of the previous year. All the contiguous buffers were aggregated to establish each year's continuous distribution; all records outside the continuous area of the previous year were considered expansion nests. From these expansion nests, those located within the new continuous area were considered to result from diffusion dispersal and those found outside this new limit were considered outposts (i.e., an evidence of jump dispersal, through either self-mediated or human-mediated).
Range expansion

To identify the range expansion trajectory, we calculated the annual increment in the continuous area. The number of new outposts per year was counted and their contribution to the overall expansion was estimated by identifying those outposts that could have functioned as a source for other nests. To ascertain a possible origin for each expansion colony and outpost, we compared its distance to the nearest edge of the continuous area and to the nearest outpost of the previous year; all the records for which the difference between both distances was lower than 5 km (corresponding to 1419 records) were discarded, being considered of non-attributable origin.

The yearly expansion resulting from diffusion dispersal along the N-S and W-E axes was estimated by measuring the distance to the south and east between consecutive limits of the continuous distribution area. The number of new nests established exclusively to the south and east from the previous continuous limit was counted and we identified how many of these were outposts. Yearly, for each outpost, its distance to the nearest source of the previous year was measured. To test for an acceleration of both types of expansion, the slopes of the relationships between these distances and year was compared with zero.

Environmental drivers

Assuming that the same variables influencing distribution have the potential to promote its dispersal, we considered three climatic and eight land cover and anthropogenic variables (see Suppl. material 1: Table S1). Variables in this study were adapted from Bessa et al. (2016), with the following changes: i) we excluded NDVI and isothermality, ii) we used the distance to each specific land cover class instead of its percentage because using distances assures better performance for landscape features (Rainho and Palmeirim 2011), iii) we incorporated the classes "distance to forest" and "index of human influence" and iv) we included predictors related to the distance to linear structures (motorways and railways). To avoid collinearity, we inspected if there were highly correlated variables (r≥0.70; Dormann et al. 2013) (see Suppl. material 1: Table S2). For this analysis we used three datasets: i) bound records (the series of points defining a minimum convex polygon that represents the leading edge of the continuous invaded area for each particular year, presumably resulting from dispersal by diffusion), ii) all outposts (resulting from jump dispersal) and iii) > 18 km outposts, representing the subset of outposts located more than 18 km (distance travelled in flight mill experiments - pers. comm., Dr. D. Sauvard, INRA, France to the authors of Robinet et al. 2017) from the continuous area of the respective year, for which there is probably a higher contribution of human-mediated dispersal.

Data analyses

To assess which variables influence the dispersal of *V. velutina* for each of the three datasets we used generalized linear mixed models (GLMM) with the package 'lme4' (Bates et al. 2015) in R (Core Team 2019). We began by running full models with climatic, land cover and anthropogenic drivers simultaneously. As climatic variables are acknowledged to be the main factors influencing the species distribution across varying spatial scales (Pearson and Dawson 2003), we decided to run additional models with land cover and anthropogenic variables only, in an attempt to find other possible predictors at a regional scale. For each dataset, we set the dependent variable as the minimum distance of the records to the continuous area of the previous year (we discarded three records that were located less than 5 km from an outpost established in the previous year, as that could be an offshoot of that outpost). To detect collinearity between explanatory variables we used the Vifstep function in the usdm R package (Naimi et al. 2014) to calculate the variance inflation factor (VIF) and excluded the variables in models with a VIF value greater than the threshold (th=3). A variable "year" was included as a random effect to account for yearly climatic variations that may affect the dispersal of the hornet. We then selected the best model (using the Akaike Information Criterion - AIC) with the dredge R function, and generated average estimates of the effect of each variable using the model.avg R function (models with delta AIC values < 2) from the MuMIn package (Bartón 2009). The results were plotted using the package visreg (Breheny and Burchett 2017).

As we verified that one anthropogenic predictor (distance to motorways; see Results) was influential on hornet jump dispersal we decided to further explore the data. First, we inspected if the outposts' distance to motorways was random, i.e., we tested whether motorways may be acting as drivers of jump dispersal. To accomplish this, we generated a twin random point for each outpost, located at the same Euclidean distance to the continuous distribution area as the outpost, and compared their distance to motorways with a paired samples Wilcoxon test. Second, for both data sets of outposts we ran another GLMM model, but this time with the distance to the entire road network to inspect the relative importance of each road category in hornet jump dispersal.

To generate a risk map of *V. velutina* dispersal and identify regions most at risk of imminent invasion, we combined information from suitable areas (regions with rainy winters and pleasant summers, mainly located along the Atlantic coast: Verdasca et al., unpublished data) with the geographical information of the significant dispersal predictors of a model with climate, land cover and anthropogenic variables (see Suppl. material 1: Table S4). These predictors were combined according to their estimates to produce a dispersal map. To define the risk areas around motorways, we analyzed the pattern of the number of outposts as a function of distance to motorways. As the number of new nests established alongside the motorways decreased linearly with distance up to 17 km from the highway (after that there was no apparent relation with distance – see Results), we calculated this relation to estimate the width of the areas that contained 50% and 75% of the outposts.

Availability of data and material

Due to privacy reasons, public data is available in Suppl. material 2: Appendix A of Supporting Information in a resolution of 5×5 km. The precise geo-localizations that support the findings of this work (which were used under license for the current study)

are available from the authors upon reasonable request and after permission of the entities holding the data. Data requests can be addressed to the corresponding author, who will make them available jointly with the different entities that hold the data. Any further information can be obtained by request to the authors.

Results

From 2013 to 2018, the area occupied by *V. velutina* in Portugal experienced a 25-fold increase (from 845 km² to 20,561.26 km²) in a linear manner without acceleration or deceleration (Fig. 1). Expansion was much faster along the north-south axis (45 km/year) than along the west-east axis (roughly 20 km/year), regardless of taking place in temperate (in 2014 and 2015) or Mediterranean climate regions (since 2016) (Fig. 1 and Fig. 2).



Figure 1. Annual expansion of *V. velutina*. The picture depicts the two cumulative linear distances (right axis) between the invasion origin and the successive limits of the invasion front to the south (black line) and east (dark grey line). The southern and eastern limits of each year were measured by simply drawing a tangent to the southernmost point and the easternmost point, respectively. The left axis refers to the yearly cumulative invaded area (in km²) resulting from diffusion dispersal and depicted as a gray area.



Figure 2. Invasion pattern of *V. velutina* in Portugal between 2013 and 2018 along the climatic gradient (TMP – Temperate climate, SP – Supramediterranean climate, MM – Mesomediterranean climate and TM – Thermomediterranean. The figure depicts the continuous distribution area of *V. velutina* in each year and the location of the outposts (points with the highest dispersal distances; see methods) by year. The suitable area for the species was retrieved from an unpublished work of the authors. The current invaded area (by May 2021) is also shown.

Range expansion

The number of outposts varied across the different years from a minimum of 4 in 2016 to a maximum of 46 in 2015. Such outposts had a very high importance for the expansion of the hornet. Indeed, the number of new expansion nests that were located near the outposts established in the previous year was higher than the number of new nests found near the previous continuous limit in all years except 2017 (Table 1).

In the first three years (2014–2016), the mean distance of new nests to the nearest outpost was lower than the distance to the continuous area (Suppl. material 1: Fig. S1). The reverse scenario occurred in 2017 and 2018, when almost all outposts were established in Mediterranean-climate regions. As the core distribution area expanded to the south and east, some outposts that gave rise to new nests nearby were engulfed into the continuous distribution area (i.e., coalescent colony model; Fig. 2).

Outposts established southwards were over 3 times more frequent than those established eastwards (Table 2). There was a decrease in the number of successfully established nests since 2016, especially southwards (Table 2). The slope of the relationship between time and dispersal distance to the south and east was not significantly different from zero, for both types of dispersal (diffusion or jump dispersal) (Suppl. material 1: Table S3). Most outposts (90%) were located more than 18 km from the continuous area of the previous year.

Environmental drivers

Models with both climatic and land cover variables explained more variability of the dispersal patterns of *V velutina* than models solely with climatic or land cover variables (Suppl. material 1: Tables S6–S8). A climatic variable – precipitation of the wettest

| Table 1. The number of new | nests and outposts of | V. velutina (outside the | e continuous | distribution | area |
|--------------------------------|-----------------------|--------------------------|--------------|--------------|------|
| of the previous year), between | 2014 and 2018. | | | | |

| Year | Number of new nests located nearer the | Number of new nests located nearer an | Total number | Number of "> |
|-------|--|---------------------------------------|--------------|-----------------|
| | continuous area of the previous year | outpost of the previous year | of outposts | 18 km outposts" |
| 2014 | 55 | 83 | 10 | 7 |
| 2015 | 418 | 491 | 46 | 46 |
| 2016 | 69 | 103 | 4 | 3 |
| 2017 | 230 | 163 | 10 | 8 |
| 2018 | 165 | 174 | 33 | 29 |
| Total | 937 | 1014 | 103 | 93 |

 Table 2. Annual number of new occurrences registered exclusively southwards and eastwards.

| Year | Number of new records exclusively southwards | Number of new records exclusively eastwards |
|-------|--|---|
| 2014 | 128 (+ 1 outpost) | 1 (+ 1 outpost) |
| 2015 | 120 (+ 23 outposts) | 330 (+ 6 outposts) |
| 2016 | 225 (+ 1 outpost) | 1 outpost |
| 2017 | 123 (+ 8 outposts) | 19 (+ 2 outposts) |
| 2018 | 24 (+ 20 outposts) | 8 (+ 3 outposts) |
| Total | 620 (+ 53 outposts) | 359 (+ 13 outposts) |

month – was the single variable selected in all the models. Temperature annual range was the only additional climatic variable identified as a driver of diffusion dispersal (Table 3, Suppl. material 1: Fig. S2, see also Suppl. material 1: Table S4 for a model with climatic and land cover variables). In the models with land cover variables only distance to shrub land (plus natural meadows) was identified as influential on diffusion dispersal; however, for jump dispersal, distance to motorways was the only significant predictor (Table 3, Suppl. material 1: Fig. S2). Distance to the entire road network (instead of distance to motorways) had no effect upon either dispersal pattern (Suppl. material 1: Table S5). In both datasets (all outposts and the subset located more than 18 km from the continuous area of the previous year), outposts were significantly closer

Table 3. Effects of distance to land cover categories and linear features (1) and climate (2) on the dispersal of *V. velutina*". Distance to the previous continuous distribution (as a proxy of dispersion) was used as dependent variable in the GLMM. The results were obtained by averaging model predictions with Δ AIC<2.

| | Multivariate model (bound records) | Estimate | Std. Error | Adjusted SE | z value | Pr(> z) | |
|---------------|--|-----------|------------|-------------|---------|------------|----|
| | (Intercept) | 21670 | 7982 | 8088 | 2.68 | 0.007 * | ** |
| | Distance to shrubs and natural meadows | 13.23 | 4.50 | 4.61 | 2.87 | 0.004 * | ** |
| | Distance to riparian galleries | -1.14 | 1.75 | 1.77 | 0.64 | 0.522 | |
| | Distance to motorways | -0.13 | 0.24 | 0.24 | 0.55 | 0.580 | |
| | Index of Human Influence | -93.37 | 171.9 | 173.9 | 0.54 | 0.591 | |
| | Distance to urban areas | -1.25 | 4.48 | 4.56 | 0.27 | 0.784 | |
| | Distance to crops | -0.03 | 0.43 | 0.45 | 0.06 | 0.951 | |
| | Distance to forest | -0.06 | 1.73 | 1.77 | 0.04 | 0.972 | |
| | Multivariate model (all outposts) | Estimate | Std. Error | Adjusted SE | z value | Pr(> z) | |
| del | (Intercept) | 54507.91 | 8590.37 | 8694.48 | 6.27 | < 2e-16 ** | ** |
| mo | Distance to forest | -30.61 | 24.87 | 25.05 | 1.22 | 0.222 | |
| ver | Distance to riparian galleries | 4.35 | 4.24 | 4.27 | 1.02 | 0.308 | |
| 3 | Distance to motorways | -0.93 | 0.29 | 0.30 | 3.14 | 0.002 * | ** |
| 1. Land | Distance to crops | -0.49 | 2.16 | 2.18 | 0.23 | 0.822 | |
| | Distance to urban areas | 0.76 | 3.55 | 3.57 | 0.21 | 0.831 | |
| | Distance to shrubs and natural meadows | -0.21 | 1.90 | 1.92 | 0.11 | 0.912 | |
| | Index of Human Influence | -0.28 | 72.54 | 73.49 | 0.00 | 0.997 | |
| | Multivariate model (> 18 km outposts) | Estimate | Std. Error | Adjusted SE | z value | Pr(z) | |
| | (Intercept) | 58370 | 8701 | 8810 | 6.626 | <2e-16 ** | ** |
| | Distance to forest stands | -16.80 | 22.77 | 22.93 | 0.73 | 0.464 | |
| | Distance to riparian galleries | 2.92 | 3.94 | 3.96 | 0.74 | 0.462 | |
| | Distance to motorways | -0.72 | 0.33 | 0.33 | 2.17 | 0.030 | * |
| | Index of Human Influence | -41.95 | 139.60 | 140.80 | 0.30 | 0.766 | |
| | Distance to urban areas | 1.14 | 4.51 | 4.56 | 0.25 | 0.802 | |
| | Distance to shrubs and natural meadows | -0.46 | 2.57 | 2.60 | 0.18 | 0.859 | |
| | Distance to crops | -0.09 | 1.24 | 1.25 | 0.08 | 0.940 | |
| | Multivariate model (bound records) | Estimate | Std. Error | Chisq | t value | Pr(>Chisq) | |
| | (Intercept) | 40882.79 | 18700.1 | | 2.19 | | |
| lel | bio7 – Temperature annual range | 188.05 | 65.92 | 8.14 | 2.85 | 0.004 * | ** |
| noc | bio13 - Precipitation of wettest month | -363.64 | 97.93 | 13.79 | -3.71 | 0.000 ** | ** |
| 2. Climatic n | Multivariate model (all outposts) | Estimate | Std. Error | Adjusted SE | z value | $\Pr(z)$ | |
| | (Intercept) | 141041.22 | 23165.9 | 23424.59 | 6.02 | <2e-16 ** | ** |
| | bio13 - Precipitation of wettest month | -678.49 | 100.71 | 101.99 | 6.65 | <2e-16 ** | ** |
| | bio7 – Temperature annual range | 31.22 | 75.37 | 76.02 | 0.41 | 0.681 | |
| | Multivariate model (> 18 km outposts) | Estimate | Std. Error | Adjusted SE | z value | $\Pr(z)$ | |
| | (Intercept) | 136405.5 | 26392.8 | 26729.3 | 5.10 | 3E-07 * | ** |
| | bio13 - Precipitation of wettest month | -630.56 | 124.99 | 126.77 | 4.97 | 7.00E-07 * | ** |
| | bio7 – Temperature annual range | 30.27 | 81.27 | 82.11 | 0.37 | 0.712 | |



Figure 3. Risk of dispersal of *V. velutina* in Portugal evidencing the buffers alongside motorways where dispersal is likely to be mostly human-mediated. The unsuitable area for the species (Verdasca et al. unpublished data) is depicted in a pale yellow. Almost all the isolated suitable areas located in the south of the country are also at risk as they are connected by motorways to other suitable regions.

to motorways than expected by chance (all outposts: 103 pairs compared, V = 1820, p-value = 0.0024 (also see Fig. 4); >18 km outposts: 93 pairs compared, V = 1306, p-value = 0.0004). We found that the number of outposts decreased in a linear function (y = -0.6103x + 10.61) with distance to motorways in a 17 km-width strip along the



Figure 4. Distribution *V. velutina* outposts (dark gray) and random points (light grey) according to the different classes of distance to motorways (km). The estimated linear function found up to 17 km is "No. nests = -0.6103 * (Distance to motorways) + 10.61".

motorways (Fig. 4 – dark grey bars). Further away, there is no apparent relationship with distance. In fact, 50% of the new outposts were located within a 6 km wide buffer zone alongside motorways and 75% up to 12 km from these linear structures.

Discussion

Major findings

The invasion of *V. velutina* is occurring at a slower pace in the northwest of the Iberian Peninsula (spread rate of approximately 45 km/year to the south and 20 km/year to the east) than in other temperate macroclimate regions (e.g., France), but faster than in other Supramediterranean climates (e.g., Italy). In the first few years of the invasion the number of new established nests was much higher near outposts than near the continuous distribution area, an indication that jump dispersal played an important role in the acceleration of the invasion process. Besides climate (namely, precipitation of the wettest month and the annual range of temperature), we found the distance to shrub lands to be influential in the dispersal of V. velutina. This finding adds new information to a previous study which also showed that land-use (namely, percentage of agricultural fields) has an important role in the expansion of this species at regional scales (Bessa et al. 2016). We also revealed that one anthropogenic driver (motorways) was important for the jump dispersal events of this flying insect, highlighting the role of these linear infrastructures in accelerating the natural invasion dynamics of *V. velutina* and the need to reinforce early monitoring programs in a 6 km wide buffer around motorways.

Range expansion

From the initial propagule found in the north of Portugal (near the coast), self-mediated dispersal has been occurring faster towards the south than towards the east. The western Iberian Peninsula encompasses different bioclimatic belts (Mesotemperate, Supramediterranean, Mesomediterranean and Thermomediterranean, Rivas-Martínez et al. 2017) that spread more along the North-South axis near the Atlantic coast than along the West-East axis. In Portugal, V. velutina is therefore faced with two transition zones, differing in extent, from temperate to Mediterranean climates, as its expansion is predominantly occurring along the Atlantic coast. To better predict the risk of invasion in the short run, it is important to disentangle the spread rate across different climatic gradients. For instance, the average rate of V. velutina expansion here identified (45 km/year to south and 20 km/year to east) is different from the one recently estimated for Portugal $(37.4 \pm 13.2 \text{ km/year}, \text{but considering all the})$ directions, and therefore an intermediate value) (Carvalho et al. 2020). We believe that it is important to refine the estimates, as the spread rate of this invasive hornet is clearly not uniform across Portugal, and this same invasion process may occur in other temperate-Mediterranean transition zones. We acknowledge that since our records are reported by citizens, a bias in V. velutina detection may be occurring due to more identifications in areas with higher population density (i.e., along the coast). However, this is a very mediatic species in Portugal, and most people are aware of this and its impacts on beekeeping, agriculture, and public health. Despite the lower population density in the eastern part of the country, there are still some important cities, numerous small villages and, even more important, more beekeepers in these rural regions. Given that we used the outermost records for each year (and not the density of records) for most of our estimates, we think that the major patterns detected, such as the differential expansion along the North-South and West-East axes, are barely affected by differences in human density. We did not find a difference in the distance of establishment of new nests between the two directions; however, there was a substantial difference in the number of new nests, as those established southwards were twice the number of those established eastwards. The climatic transitions are more abrupt towards the east, where the hornet is now facing Mediterranean climatic conditions (i.e., drier, higher range of temperatures), which may explain why the species has more difficulty in establishing new nests in this direction. This may be the reason for the decrease in the numbers of established nests since 2016 and supports the importance of climate for the expansion rate of this hornet. In fact, the expansion area is not increasing exponentially as would be expected if diffusion was occurring equally across all directions. In France, the species spread rapidly toward the northeast and not so much to the south (Robinet et al. 2019). In Portugal, the rate of expansion was lower in 2018, potentially due to the major and uncontrolled wildfires that occurred in 2017 precisely over the distribution limit of V. velutina in that year. Indeed, by December 2019, the spread rate towards the south was again near 50 km/year (see http://stopvespa.icnf.pt/ by ICNF; also check the invaded area

in Portugal by May 2021 in Fig. 2). Extrapolating to other temperate-Mediterranean transition zones, such as the Italian and Balkan peninsulas, the rate of expansion and invasion pattern may be similar.

Roles of diffusion and jump dispersal

As in other countries, V. velutina in Portugal is dispersing by both diffusion and jump dispersal. This same pattern was noticed in France (Robinet et al. 2017) and Italy (Bertolino et al. 2016; Lioy et al. 2019), as well as for other social Hymenoptera (like the Argentine ant; Suarez et al. 2001). The frequency and distance of jump-dispersal events are thought to be stochastic, and therefore difficult to predict. For species that spread through stratified diffusion, the distance and rate at which new foci are created through jump dispersal may be more important than the rate of spread through diffusion from established foci (Suarez et al. 2001). Here, the successful long-distance dispersal events played an important role in the expansion of V. velutina in almost every year after its establishment, as the number of new nests was higher near outposts than the boundary of the continuous distribution area (Suppl. material 1: Fig. S1). These results support a coalescent colony growth model, similar to prior studies that found outposts to accelerate range expansion (Shigesada et al. 1995). Previous studies from Italy (Bertolino et al. 2016; Lioy et al. 2019) found the dispersal of V. velutina to be hindered by high mountain ranges (above 700 m), and therefore argued that this may be one of the main reasons for the low spread rate in Italy (18 km/year: Bertolino et al. 2016) compared to France (78 km/year: Robinet et al. 2017). Spread rates similar to those in Italy were registered in Korea (10-20 km/ year), although the low spread rate there may be due to competition with six other hornet species (Choi et al. 2012). Nonetheless, the constant spread rate observed so far in Portugal may begin to decrease southwards when the species reaches Mediterranean climates. Although all outposts were located within the suitable area for the species, V. velutina is not (yet) in geographical equilibrium, since, according to our former work, there are still suitable areas to the south and east (Verdasca et al. unpublished data). This apparent limit on the establishment of outposts corroborates our estimates about the adequate areas for the species; however, as the species reaches its estimated limits, it is important to assess how robust they are, as the colonization of adjacent areas, or even the adaptation to novel environmental conditions is possible. If these limits hold, this means that jump dispersal will be the only dispersal mechanism allowing the species to reach the isolated suitable areas in the south of the country.

Influential environmental attributes on the direction and speed of V. velutina dispersal

The dispersion of *V. velutina* is affected by precipitation and temperature gradients, a result that is similar to those of other studies that modeled the hornet's bioclimatic niche (Villemant et al. 2011; Verdasca et al. unpublished data). Besides precipitation of the wettest month and the annual range of temperature, distance to vegetated, but treeless landscapes (covered by shrubs and natural meadows) seem to favor diffusion dispersal. As shrub lands

provide a wide variety of nesting sites and food resources for wild pollinators (Chaplin-Kramer et al. 2011), hornets will probably need to fly over longer distances until such pollinator suitable habitats can be reached. In regions that are climatically suitable, the presence of shrubs may thus reduce hornet dispersal. However, shrub land cover is probably not related to the large difference between the eastward and southward rate of expansion, as this habitat is regularly found across the suitable area for the species in Portugal.

Precipitation in the wettest month, and motorways, were the only factors identified as drivers of jump dispersal, but the role of motorways in the dispersal of the hornet was only detected when the climatic predictors were not included in the models. This is in line with the scale dependencies outlined by Pearson and Dawson (2003) – different processes are more important at different scales i.e., at a continental scale, climate can be considered the dominant factor, whilst at more local scales factors including topography and land-cover type become increasingly important. Further down the hierarchy, if conditions at higher levels are satisfied, factors including biotic interactions and microclimate may become significant (for details on hierarchical modeling framework see Pearson and Dawson (2003)).

Motorways facilitate jump dispersal

The fact that motorways were important predictors of outposts is an indication that they may have resulted from human-mediated dispersal. Yet, as motorways are heavily used by people, a potential bias in the detection of nests near these human infrastructures may have occurred. However, most motorways pass through remote places with low population density, and people cannot stop their cars over vast extensions. Therefore, it is unlikely that nest reports come from people using the motorways. Jump dispersal events were predicted by motorways, but not by all roads, railways, or the index of human influence, a variable highly correlated with human population density (e.g., cities). This is an indication that the establishment of outposts is probably mediated through the movement of vehicles and goods, such as wood products and bark or man-made goods (e.g., ceramic pottery associated with garden trade), which in Portugal occurs mostly through the motorways. These products provide suitable refuges for hibernating inseminated V. velutina queens (Marris et al. 2011); indeed this was the most probable route of incursion of *V. velutina* in Europe, on pots imported from coastal China, near Shanghai. However, it is also plausible that a dispersing gyne may simply land on a car that then travels a good distance away and starts a nest there. As V. velutina gynes can fly over long distances and generate stochastic patterns of spread similar to those resulting from human-mediated dispersal (Robinet et al. 2017), it is probable that some of the records may have originated from self-mediated dispersal. However, the overall detected effect of motorways regardless of the distance group considered, together with the decreasing trend in nest abundance as one proceeds away from the motorways, are difficult to explain through self-dispersal alone.

The association of nest establishment with motorways was only found for outposts (50% and 75% of them established within a 6 km and 12 km wide buffer zone alongside motorways, respectively), and not for records that originated from diffusion dispersal. Our findings corroborate a previous study in Italy (Porporato et al. 2014)

where a high number of the observations and captures of *V. velutina* in bait-traps were recorded near highways, emphasizing that freight traffic can contribute to the transport of this species far from the invasion front. The fact that Bessa and collaborators (2016) did not find any relation with the road network could be due to the very restricted region that was used in their study (roughly 10% of the area that we used here). In France, Robinet et al. (2017) used human population density as a proxy for trade to test jump dispersal, not taking into account the road network, and concluded that the rapid spread of the hornet may not be necessarily mediated by humans. So, it is possible that long-distance dispersal events that occurred in France may also have contributed to unintentional introductions via motorways.

Despite it being extremely difficult to provide evidence for early introductions, other social insects have also probably been transported accidentally by humans over long distances since the establishment of long-distance trade routes (Bertelsmeier 2021). For example, New Zealand had no social wasp species prior to human colonization, but over the last century has been invaded by several species of social wasps (Lester and Beggs 2019). Indeed, Vespula germanica and Vespula vulgaris, both native from Eurasia, have become widespread throughout the New Zealand causing major impacts to native biodiversity (Lester et al. 2014). In Argentina, where V. germanica is also invasive, the observed stratified geographical expansion pattern (which frequently exceeds 30 km per year, although faster to south) does not match the observed queen dispersal abilities (only a few hundred meters naturally to find nest sites), suggesting that human-aided transport of hibernating queens is the central driver of the current distribution of these wasps in the country (Masciocchi and Corley 2012). At more local scales, the anthropogenic influence on the spread of invasive insects was also demonstrated. For example, the distance to railroad tracks influenced the spread of the invasive termite species Reticulitermes flavipes (Perdereau et al. 2019).

Implications for the management of Vespa velutina invasion process

Identifying pathways that facilitate the dispersal of invasive species is essential for informing efforts to contain invasions (Suarez et. al. 2001). To be successful, every invasive species control program must consider the probability of detecting the species and the cost of the process. In this work, we showed that 50% of the presumed new nests resulting from human-mediated long-distance dispersal established within a 6 km wide buffer along motorways. To raise this proportion to 75%, the buffer must be increased to 12 km, which represents an almost 70% increase in the area to be surveyed. Based on results here, effective measures to contain *V. velutina* invasions should include early monitoring programs in a buffer of 5 km (the maximum homing ability that few hornet workers can reach: Poidatz et al. 2018) around the continuous distribution area of the previous year, and 6 km (ideally 12 km) around motorways. If the climatic conditions are met, the vicinity of the main roads is susceptible to be colonized faster through human-mediated transport. Even in highly fragmented habitats, the main roads can connect isolated suitable areas. For instance, in the regions at risk in southern Portugal, the area to be surveyed can be limited only to climatically favorable regions that are reachable by highway. This is

particularly relevant in southern Portugal where the isolated fragments of suitable landscape are economically very important for beekeeping activities. The early detection and control of nascent populations in these areas may be a good way to manage its spread, rather than focusing efforts on established invasion fronts. Local outreach activities, especially those targeted to transportation companies, should also be prioritized to prevent the European motorway network from becoming an invasion route for the hornet to new countries. However, different types of cargo do not carry the same risk of being infested (as different species may differ in their commodity associations). Therefore, focused biosecurity policies for *V. velutina*, are needed, particularly targeted to the interception of wooden products' transportation and man-made goods associated with garden trade, due to the potential of these commodities to shelter hibernating queens. It is also important to promote control actions on ports of species entry, namely harbors along the coast.

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Supplementary material I

Supporting information

Authors: Maria João Verdasca, Hugo Rebelo, Luísa G. Carvalheiro, Rui Rebelo Data type: tables and figures (pdf. file)

- Explanation note: Table S1. Climate, land cover and anthropogenic variables with potential to affect the behaviour and establishment of Vespa velutina. Tables S2. Correlation matrix of the climatic, land cover and anthropogenic drivers that have the potential to affect the behaviour and establishment of *Vespa velutina* in Europe: a) bound records; b) outposts; c) outposts >18 km. Table S3. Relation between dispersion distance (to south and east) vs time either for self mediated or jump dispersal by testing through t-test the significance of the slope of the regression when compared to zero (H0: the slope of the regression line is 0). Table S4. Effects of climatic, land cover and anthropogenic variables on the dispersal of V. velutina. Table S5. Effects of the different land cover and anthropogenic predictors on the dispersion of V. velutina, considering the predictor distance to the entire road network, instead of distance to motorways in bound records and both sets of outposts. Table S6. Set of best models with climatic and land variables according to the different datasets (1. bound records; 2. all outpost and 3. outpost 18 km). Table 7. Set of best models with climatic variables according to the different datasets (1. bound records; 2. all outpost and 3. outpost 18 km). Table S8. Set of best models with land variables according to the different datasets (1. bound records; 2. all outpost and 3. outpost 18 km). Figure S1. Variation of the mean distance of the new records within a given year to the nearest potential source: continuous area or outpost. Error bars depict standard errors. Figure S2. Relation between the dispersion of Vespa velutina and significant climatic and land cover variables according to the different datasets: bound records, all outposts and outpost 18 km (see Table 3 of the main manuscript).
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Supplementary material 2

Appendix A

Authors: Maria João Verdasca, Hugo Rebelo, Luísa G. Carvalheiro, Rui Rebelo Data type: occurrences (pdf. file)

Explanation note: Vespa velutina occurences - 5 km resolution.

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