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METHODS

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A low-cost, durable, submersible light trap and customisable LED design for pelagic deployment and capture of fish parasite *Salmincola* sp. copepodids

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Abstract

Documenting species invasions and assessments of ecological changes depend on detection. Here, we present a simple design of a plankton light trap with specific wavelength LEDs and modifications. We used PVC pipe to create standardised small, rigid, low-cost traps that can be deployed in lentic habitats. With a cost of under $30 US each, including lights and rechargeable batteries, our traps are affordable without the need for disposable chemical lights. These small traps rely on a vacuum to retain contents upon retrieval, eliminating complicated closing mechanisms and allowing bottom entry. Our design includes submersible LED lights that can withstand pressures of at least 5 atm. We expect that the included instructions for underwater light construction and rubber weights using sand may be broadly applicable. However, we designed and field-tested our traps focusing on the detection and capture of the infective copepodid lifestage of a freshwater parasitic copepod, *Salmincola californiensis*. This lifestage had previously only been observed by rearing in a laboratory setting and is of concern due to continued spread outside of its native range and detrimental impacts on salmonids, especially in freshwater reservoirs. We used a 445–450 nm wavelength LED light for capturing *Salmincola* copepodids, but the light design can be modified to any readily available LED and heat sink to attract other target organisms. In our case, the overall affordability of the trap and components allowed for the extensive trapping needed to capture and map the occurrence of rarely-observed species and lifestages, such as the copepodids of *S. californiensis*. In general, increasing the number of traps that can be deployed within or across sites can aid in the spatial comparisons of plankton distributions needed in studies of ecology and species life histories. Light traps may aid in the detection of introduced zooplankton, such as *S. californiensis*, outside of their native range and associated plankton community changes.

Keywords
lentic, parasitic copepod, pressure, reservoir, *Salmincola californiensis*, salmon, zooplankton

Introduction

Zooplankton are integral to the ecology of aquatic habitats (Lehman 1988), including free-living holoplankton species as well as juvenile life-stages of invertebrates and vertebrates that transition to non-planktonic adult forms. Introductions of both free-living and parasitic zooplankton can have important ecological implications (Yan et al. 2002; Havel and Shurin 2004; Duggan and Pullan 2017). In many cases, zooplankton lifestages contain critical transitions such as host-finding (Heuch et al. 2007) or settling (Freckelton et al. 2017) that are often poorly understood outside of laboratory settings because of the difficulty of field collection. For species of concern, collecting infectious and larval stages from the wild may be important in understanding population dynamics and detecting novel invasions (Suárez-Morales and Mercado-Salas 2013). However, such collections can be challenging in environments that are difficult to sample by tows and when taxa have strong swimming abilities or are otherwise under-represented when using traditional netting collection methods (De Bernardi 1984; Porter et al. 2008).

Many plankton species, especially those with strong swimming abilities, may be phototactic in order to regulate their position in a water column (Martynova and Gordeeva 2010). This can reduce predation risk while maximising foraging opportunities for planktonic consumers that exhibit diel vertical migration, with light acting as an external cue and providing directional orientation (Forward 1988). For organisms exhibiting positive phototaxis, light traps are a logical method of collection (McLeod and Costello 2017). Light trap designs vary widely, but include a light source and an opening that narrows towards the interior of the trap (McLeod and Costello 2017). Often, traps have horizontal entrances (Meekan et al. 2001; Fisher and Bellwood 2002; Hernandez and Shaw 2003) with collection into a mesh cup or basket at the bottom of the trap. Traps are often mesh-sided; however, mesh nets may experience problems with clogging during phytoplankton blooms (Jones 1971) and by non-target animals attempting to enter the trap (McLeod and Costello 2017). Traditional light traps are often expensive and require construction by a machine shop (Floyd et al. 1984). Recent efforts towards inexpensive light traps include modifications of existing water samplers or nets and the use of disposable light sticks (Kehayias 2006; Kehayias et al. 2008), as well as larger designs that incorporate inexpensive materials with a modular light box and fluorescent lighting (Watson et al. 2002). Light traps can be quite large, up to one metre in height (McLeod and Costello 2017), though smaller traps may be as or more effective (Meekan et al. 2001). Designs for small, simple, inexpensive traps and light source customisation to collect phototactic plankton are lacking, especially considering the recent broad availability of selective wavelength LED components.

Light traps may capture species that are absent in other sampling efforts (Jones 1971). For example, *Salmincola* sp. are freshwater Lernaeopodid copepods, known as natural parasites of Brook Trout and other salmonids (Fasten 1912). However, these copepods have been recently recognised as a problem in regions where they have likely been introduced along with host fishes (Kamerath et al. 2009) and in altered systems including reservoirs (Hargis et al. 2014; Monzyk et al. 2015; Lepak et al. 2021). *Salmincola californiensis* is of particular concern because of high infection prevalence and parasite burden on threatened Chinook Salmon (*Oncorhynchus tshawytscha*) and associated impairment of the fish in Oregon, USA (Herron et al. 2018; Neal et al. 2021). Yet, copepodids (the infectious, free-swimming lifestage) of this species were not detected after four years of extensive tow netting in multiple upper reservoirs of the Willamette Basin (Murphy et al. 2019a) where *Salmincola* sp. adults were common on salmon (Monzyk et al. 2015). Unlike sea lice (family Caligidae), *Salmincola* sp. (commonly known as gill maggots) permanently anchor onto their host during development. Their development includes a non-swimming nauplius, a motile copepodid and attached chalimus and adult stages and they are restricted to freshwaters throughout all lifestages (Kabata and Cousens 1973; Murphy et al. 2020). Although adult female *Salmincola* sp. are easily observed on parasitised fish, the infectious copepodid stage has not been captured in the wild. Rather, this stage was described in the laboratory through hatching egg sacs from adult females (Kabata and Cousens 1973). Capturing copepodids could provide early detection where sensitive fish are rarely handled, offer insight into the conditions that are exacerbating infection rates and may eventually lead to methods for management and control. There remain challenges to capturing and quantifying species exhibiting primarily vertical (not horizontal) movements, such as copepodids of the parasitic genus *Salmincola* (Poulin et al. 1990) and to producing a sufficient number of traps to detect their presence and distribution (Doherty 1987), assuming they may be patchy in space and/or time (Herron et al. 2018).

To figure out how we might capture *S. californiensis* copepodids, we conducted extensive laboratory observations and revised the literature about sampling for infectious stages in other groups of parasitic copepods. We noted, as had previous studies for related species, that *S. californiensis* copepodids were negatively buoyant and frequently rested on the bottom of laboratory tanks outside of active bouts of swimming (Poulin et al. 1990). Based on the findings of Penston et al. (2004, 2008) for sea lice, we initially focused on mid-water tows and benthic sleds for copepodid capture. However, such sampling failed to yield copepodids from reservoirs where high levels of infection were documented (Monzyk et al. 2015). After noting positive phototaxis exhibited in the lab and the design by Novales Flamarique et al. (2009), we focused on the design and construction of customised light traps as we did not find replicable published designs that would allow capture from below (but see Tranter et al. 1981). Generally, leaving openings at the bottoms of traps would allow water (and captured material) to flow out of traps during retrieval unless there was a closing mechanism. Noting the limited information available on the customisation of light sources, on designs for traps with bottom openings (important for captures during the process of vertical migration) and on designs minimising mesh (important for species that are easily damaged or difficult to remove and to avoid clogging during phytoplankton blooms), we designed a novel small, robust trap with customisable lighting that can be run in vertical transects. A non-mesh trap was important because of the presence of structures and debris (e.g. stumps) that could damage mesh traps, as well as phytoplankton blooms that cause clogging of mesh cod ends. Designs using acrylic that fit these criteria were cost prohibitive for the extensive trapping we anticipated for this difficult-to-capture infective lifestage of the parasitic copepod. Finally, the commercialisation of designs for aquaculture has limited the information available to guide light trap construction. Here, we describe how we built, set and collected samples from our low-cost, robust light traps that can be deployed in series from the water surface to depths of at least 60 m and that can be outfitted with selective wavelength LEDs, based on target species. We also provide field test data from three reservoirs in the upper Willamette River Basin in Oregon, USA, to demonstrate the efficacy of these traps.

Methods

We captured *S. californiensis* copepodids from the wild (Cougar Reservoir, OR, USA, November and December 2018) using our novel light traps. These first captures of *Salmincola* sp. relied on prototype traps constructed using clear, glass funnels set into the bases of 5-gallon (19 l) buckets with sealed lids. Although successful, these traps were large and easily damaged. During associated field trials, many of the underwater lights we tried were ineffective, not modifiable and cost prohibitive. Our study sites are used for human recreational purposes and valuable equipment is more likely to be disturbed or removed. Thus, we explored PVC-based traps to focus on improving portability and durability while maintaining a cost that would be reasonable to deploy at a large scale and that could accommodate loss. The final trap design is based on an 18 cm length of 4-inch (10.16 cm) diameter Schedule 40 PVC pipe that is easy to source in the plumbing section of local hardware stores (Suppl. material 1: Table S1). We were unable to reproduce the closing mechanism in the trap described by Novales Flamarique et al. (2009), even after contact with the authors, so we focused on a simple design that would create a vacuum, thus retaining contents upon retrieval. Lights were placed in water-tight jam jars and either secured to the top lid or secured at the top of the trap by having their latch seated in a cut-out at the top of the pipe (Fig. 1). The light beam was directed downwards through a glass funnel placed in the bottom lid. We designed the traps to use rechargeable AA batteries because they are easy to source and minimise disposable waste after intense use.

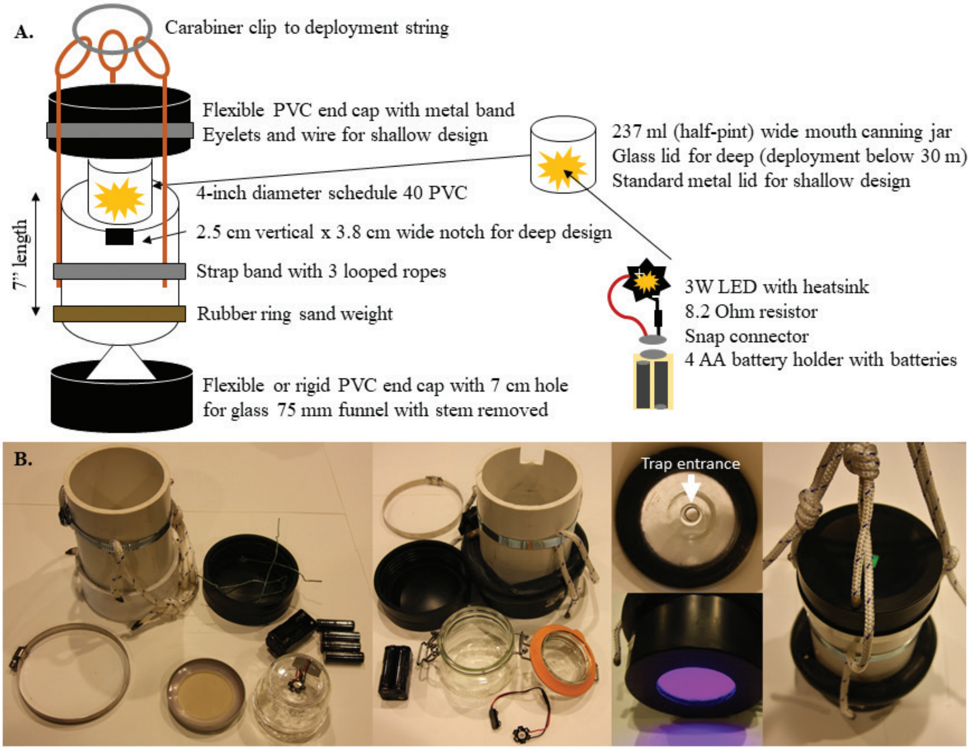

Figure 1. Trap design **A** trap schema and component placement. Light is placed under the lid facing down towards the open inward-facing funnel at the bottom (entrance 8 mm at narrowest point, 75 mm diameter at the widest) **B** views of traps including standard (left), deep (centre) and funnel, light colour and deployment loops (right). The rubber weight is used on both trap configurations to ensure vertical orientation, but is pictured on the deep trap here, for reference.

Trap assembly

Simple solid selective wavelength LED

We constructed our lights using LEDs that came already affixed to a heatsink. This meant that connections were simple solders of the positive and negative connector wires from the battery snap connectors to the heatsink. We spliced a resistor into the negative wire, although it could be spliced into either wire, and covered the resistor and associated solder connections with heat shrink (Fig. 1). The lights were stored in their respective jars and snapped to the battery holder during deployment. We chose a resistor that would provide more than 48 hours of relatively consistent bright light, based on the battery discharge curve for the rechargeable batteries we used.

Optional microcontroller and light array

Our initial trials in the laboratory used lights of differing wavelengths and a microcontroller to adjust intensity and mimic shadows. We describe that version here, although we did not complete field testing using it because solid lights were more effective for our target species during lab comparisons. These instructions should aid in the design of more specialised lights and the code (Supplement) can be adjusted to control individual light parameters. Prototype photos, firmware code and a shopping list for the microcontroller array can be found in Suppl. material 1: Fig. S2.

Trap body (PVC cutting, optional notch and securing lines)

For the trap bodies, we cut PVC pipe into ~ 18 cm long pieces using a mitre saw. This was all that was necessary for traps to be deployed in shallow water (< 30 m). For the traps to be used in deep water (≥ 30 m), we cut a 2.5 cm deep by ~ 4 cm wide notch into the top perimeter of those trap bodies. We marked the opening and cut downwards using a hand-held jig-saw. Channel lock pliers were used to bend the newly-created tab of PVC inwards and snap it off. Notches allowed seating of the rear hinges of the snap closures of glass-lidded jars; we wedged the front latch of the closure fully into the trap body when traps were set. Care was taken not to cut the notch too deeply, as the flexible PVC top lid must seal fully below the notch. Further, we prepared traps for deployment by securing three ~ 55 cm lengths of rope to the trap body with a worm gear clamp. We tied a triple sliding hitch in each length of rope so that fine adjustments to vertical balance could be easily made in the field.

Lower lid preparation

When flexible PVC lids were used, they were placed on the PVC pipe to stretch prior to construction to minimise issues during assembly and deployment. Lower lids varied in material, but we took care to minimise the edge spaces where trap contents could become lodged and filled any remaining gaps with silicon for the rigid PVC or ABS lids. This extra care was not necessary with flexible PVC lids. Regardless of bottom lid material, we used a drill press with a 7 cm hole saw to make a perforation in the centre of the lid. We attached the lid to the PVC pipe by either hammering it in place (rigid) or by placing it on and securing it with a worm-gear hose clamp tightened with a screwdriver (flexible PVC).

Funnels were prepared by cutting the stem off (before the opening widened) using a hand-held metal file (scoring the stem on all sides). We then lined the large end of the funnel opening with silicone (for adhesion) and firmly placed the funnel inside on the lower lid of the trap to cover the hole in the lid and ensuring a complete seal around the funnel perimeter. We left the trap open and undisturbed for 24 hours to allow the silicone to cure and the funnel to be fully secured.

Top lid preparation

Top lids were always the type made from flexible PVC. The top lid was used without modification for traps for deep deployment that used glass snap-top jars, because the jars were held at the top of the trap by the notch in the PVC for their closing mechanism. Standard, metal-top jam jars used for traps deployed in shallow water needed to be secured to the top lid. For this, we used four small eyelet screws placed evenly spaced just inside of the ring where the PVC tube is seated when the cap is on. We threaded one wire piece through each screw and secured the wire with pliers. We then bent the wires around each other by hand when loading the light jars during deployment

Weights

We cut used bicycle inner tubes into sections of approximately 55 cm, discarding the stem valve and pieces with large holes. We sealed one open end of the tube by folding it over and securing it with duct tape. Then we used a funnel to fill the tube with sand. We left ~ 8 cm of the tube unfilled and looped the tube around a length of ~ 10 cm diameter PVC so that the open, unfilled section overlapped the sealed end. We then wrapped the two ends together with tape to seal and secure the weight in a circular form of ~ 12.5 cm diameter. The weights stretched, so there was flexibility in sizing. Bike tube diameters also varied, but weights were only used to keep traps orientated vertically, so exact weighting was not necessary.

Deployment line features

To allow setting of multiple traps along a vertical transect, we prepared loops at pre-measured distances along a deployment line of a measured length (we made loops at 1 m, 2.5 m, 5 m, 10 m, 15 m and 20 m along a 25 m line) that was also looped at both ends. Loops at the top and bottom of each line were used for attaching an anchor and a buoy. We ensured that butterfly loops along the line were sized to allow the weighted traps to be positioned next to the line without being forced at an angle (e.g. > 9 cm). Anchors were made by adding water to concrete mix and pouring the concrete into ~ 15 cm lengths of concrete form tube (placed on disposable plastic or cardboard). We placed one eyebolt into each concrete anchor, ensuring the bolt was in place and jiggling the mould after placement to settle the concrete around the bolt. Anchors were left to dry for approximately one week, though curing was weather dependent. Additional lines were prepared in pre-measured spools to link deployment lines to buoys when waterbody depths were greater than the length of the deployment lines.

Trap deployment

We placed a light (facing down) in each jar and secured it into position with a small piece of tape. Then we put batteries into holders and snapped the holders to the light contacts. Lights were placed in either jars sealed with a silicone ring, metal lid and metal band for shallow deployment (< 30 m) or snap-top jars with glass lids and rubber gaskets for deep deployment (≥ 30 m). The glass-lidded jars were needed for deep deployment because metal jar lids crush from the pressure at depths of more than 30 m. Note: batteries should not be added early to unclipped battery holders, especially in metal-lidded jars, to avoid a risk of shorting.

Jars were fixed into position in the traps either by twisting the wires attached to the lid (shallow) or by being seated into the notch cut into the trap body (deep). We filled a tote with filtered water that we used to fill the traps prior to deployment. After filling each trap with water by submerging it in the tote, we secured the top lid to each trap while submerged and used a screwdriver to tighten the associated clamp, making sure that the clamp held the lid tightly to the trap body with a complete seal. We then clipped each trap to a loop in the deployment string as we lowered the string and associated anchor into the water.

Trap collection

At each vertical transect, traps were collected sequentially from surface to bottom and placed into white primary wash basins (11.4 l) upon retrieval. These were labelled with the depth for each trap. Once trap lids were loosened and the vacuum was broken, contents flowed into the small primary basin. Wash bottles were used to thoroughly clean out each trap and wash any additional organisms into the wash basin. The basin contents were then sieved using a 106 µm test sieve and washed through a funnel into a collection bottle (250 ml HDPE Nalgene). Primary basins found to contain juvenile fish were nested inside of larger (17 l) secondary basins containing ice for at least 20 min to anaesthetise the fish. All samples were preserved using 95% ethanol with a final concentration of at least 75%.

Field assessment

We set our light traps monthly from June through to December 2019 at three reservoirs (Cougar, Fall Creek and Lookout Point) in the Upper Willamette Basin, Oregon, USA. From June through to September, we set five vertical strings at each reservoir (Fig. 2) and, on each string, we typically set traps at distances of 1, 2.5, 5, 10, 15 and 20 m from the bottom of the reservoir. Fewer traps were set on a string if water depth at a sampling location was less than 20 m. Similarly, from October through to December, we set five strings per reservoir, but on each string, we set traps at 1, 2.5 and 5 m from the bottom and set additional traps every five metres from the bottom to the surface regardless of the depth at the sampling location, thus adding a few more traps per string at the deepest locations. However, because one of the reservoirs (Fall Creek) was drained to the stream bed before sampling in November as part of normal operations for that site (Murphy et al. 2019b) and the reservoir only partially refilled

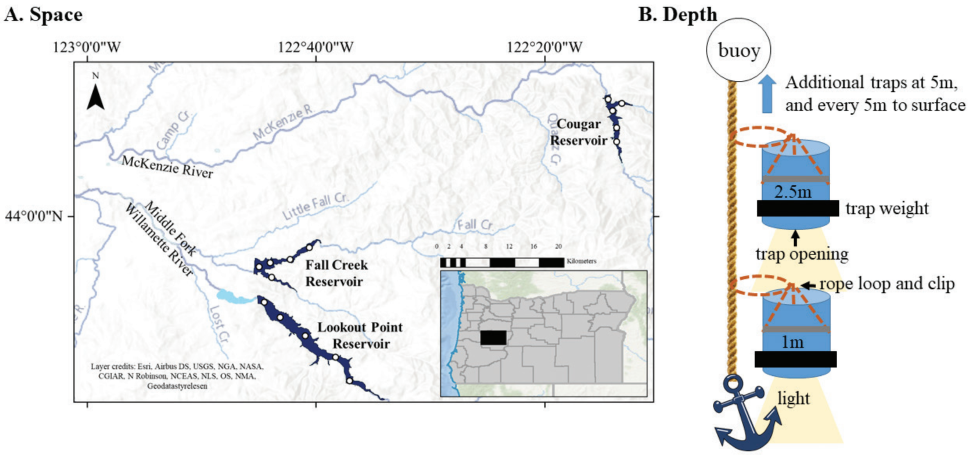

Figure 2. Representation of field deployment set-up over **A** space and **B** depth within reservoirs in the Willamette Basin, Oregon. Traps were placed at five sites per reservoir (as indicated with white circles) with traps at 1, 2.5 and 5 m from the benthos continued by traps at 5 m intervals to the surface along a single vertical deployment string held in place by an anchored buoy. Traps were clipped on to pre-tied loops in the deployment rope (represented as dashed orange ovals) to facilitate placement.

in December, fewer strings and traps were deployed at that reservoir in the last two months of sampling.

For our field test, each month, we deployed traps for 48-hour and lights continued to operate throughout deployment. We chose to use 445–450 nm wavelength LED lights in our traps because laboratory trials we conducted showed these were better at attracting *S. californiensis* copepodids than white LEDs or LEDs producing narrow bands of longer or shorter wavelengths (Suppl. material 1: Table S2). The lights we used were set to be always on and not fluctuating in intensity because laboratory trials indicated this was more attractive than when lights fluctuated or operated in series to simulate shadows of passing fish (Suppl. material 1: Table S2). Nevertheless, we provide a microcontroller design as an option for variable light settings in Suppl. material 1:Figs S1, S2 in case that could be useful in other applications and for other species. We added bait to our light traps to increase the probability of capturing target organisms (Burkett et al. 2001). We hoped scent from the addition of salmonid fish tissue would increase the attraction of light traps to the parasitic *S. californiensis* copepodids, but would decrease the attraction of the light traps to non-target organisms that are preyed upon by fish. Inclusion of fish tissue in the traps was accomplished by placing some frozen salmon fin tissue in a teabag and positioning that between the lid of the light jar and the top lid of the trap in each trap. Although we did include bait in the traps for our field test, our laboratory trials indicated that this type of bait did not affect the attraction of light traps to *S. californiensis* copepodids, but it did slightly reduce captures of non-target free-living zooplankton (Suppl. material 1: Table S2).

Field-collected light trap samples were examined in the laboratory as described below. Further, for comparison with light traps results, we also collected monthly vertical tow samples and Van Dorn trap samples at each reservoir (see Murphy et al. 2019a for tow methods and analytical procedures).

Laboratory methods

Light trap samples were processed in the laboratory to identify and count *S. californiensis* copepodids and other non-target organisms that were also captured. To increase our efficiency at searching for *S. californiensis* copepodids, we washed each sample through a pair of stacked sieves (Fig. 3A). The upper sieve had a 500 µm mesh size which retained mature *Daphnia* spp., large calanoid and cyclopoid copepods and other large organisms (e.g. fish larvae, *Leptodora* sp. and aquatic insects), but allowed *S. californiensis* copeopodids and smaller zooplankton to pass through (Fig. 3B); the lower sieve had a 106 µm mesh size to retain the size fraction of zooplankton that included *S. californiensis* copepodids (Fig. 3C). We washed plankton retained on the mesh of the 106 µm sieve into a Petri dish with 95% ethanol and searched this

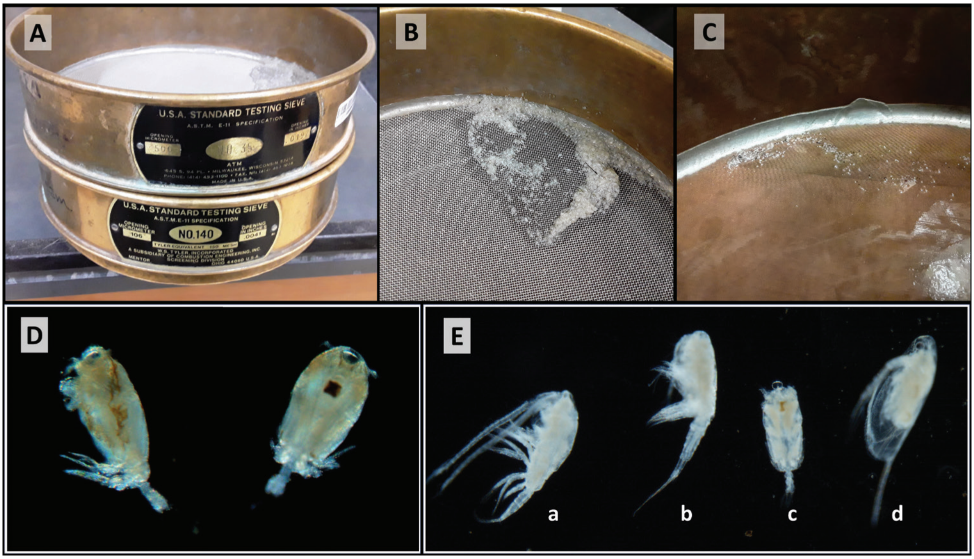

Figure 3. Photos of sample processing and characteristics of *S. californiensis* copepods compared to other common zooplankton in light trap samples **A** stacked sieves used in light trap sample processing **B** large zooplankton retained on the 500 µm sieve **C** smaller zooplankton that could include *S. californiensis* copepodids retained on the 106 µm sieve **D** freshly preserved *S. californiensis* copepodids from a laboratory rearing experiment **E** comparison of common zooplankton caught in a light trap and retained on a 106 µm sieve including: a) a calanoid copepod, b) a cyclopoid copepod, c) a *S. californiensis* copepodid and d) a *Daphnia* species.

material thoroughly under a dissecting microscope at 8X-20X magnification. Any *S. californiensis* copepodids found were recorded and removed from the sample.

In practice, distinguishing *S. californiensis* copepodids from other zooplankton was not difficult. We had examined many of these copepodids during laboratory hatching and rearing experiments (Murphy et al. 2020) and there are excellent illustrations and descriptions of the morphology of *S. californiensis* copepodids in Kabata and Cousens (1973). We identified other organisms in the light trap samples using keys and illustrations in Thorp and Rogers (2016). In comparison to free-living copepods, *S. californiensis* copepodids have a different body form including a lack of extended first antennae modified for swimming, a transparent lens-like structure at the anterior end of the body (the terminal plug of the frontal filament used to attach to a host fish) and a pigmented eye spot containing three ocelli in the middle of the dorsal surface of the anterior portion of the body (Fig. 3D, E). The pigment of the eye spot, however, can fade somewhat with prolonged storage in ethanol preservative.

After searching for and removing *S. californiensis* copepodids from each light trap sample, we drained the alcohol from the remaining material in the Petri dish using the 106 µm sieve and rinsed this material and the material retained on the mesh of the 500 µm sieve into a beaker with water; a couple drops of detergent were added to prevent organisms from clumping. We then subsampled these non-target organisms using a Folsom plankton splitter to target 200–400 organisms for representative identification and enumeration.

Results

We successfully captured *S. californiensis* copepodids using our light traps, but did not detect them in concurrent tow or Van Dorn traps samples (Table 1). In total, we collected 532 light trap samples across all reservoirs and sampling dates and 53 of the traps captured our target organisms. For the traps that captured *S. californiensis* copepodids, the number of copepodids captured ranged from 1 to 169 with a median of 2. Non-target organisms were also captured in our light traps (Suppl. material 1: Table S2). Most of them were cladocerans (primarily *Daphnia* spp.) and calanoid and cyclopoid copepods. Other organisms caught in lower numbers were various aquatic insects, aquatic mites, ostracods, amphipods and larval fish. Captures of all organisms in light traps were highly variable depending on reservoir, collection date and trap depth. Interestingly, *Salmincola* copepodids were captured at shallower and warmer conditions than expected, based on documented copepodid survival (Murphy et al. 2020; Suppl. material 1: Table S2).

Discussion

Here, we describe light traps that successfully attract and capture *Salmincola* copepodids from lentic systems with the potential to be used to examine patterns of both spatial and vertical distribution of this species in our study reservoirs. In order to sample for this 'needle in a haystack', we present a robust low-cost trap design that can be widely deployed, including at depths with pressures of 5.9 bar and in environments that could damage mesh-based traps (e.g. reservoirs with large stumps and woody debris). The simple design eliminates the need for complex and expensive closing mechanisms and is constructed from easily-obtained materials. By establishing methods to capture this poorly-understood copepodid life stage, we will be able to gain valuable ecological knowledge about its density and distribution over time and space. This information should help us to understand the current problem of high infection prevalence and intensity and may allow us develop remediation strategies. In addition, the use of our light traps provide monitoring data to prompt ecological discoveries (e.g. use of optimised traps as potential method of control and management of copepodids).

Table 1. Number of light traps that captured *Salmincola californiensis* copepodids in our field trial at three upper Willamette Basin reservoirs. Samples were collected monthly from June through to December 2019. Number of traps set given in parentheses. Note: corresponding tow net samples from 35 m to surface (0.5 m diameter, 64 μm) failed to capture copepodids during the study period.

Reservoir	June	July	August	September	October	November	December
Cougar	0 (25)	1 (29)	3 (28)	10 (28)	7 (33)	7 (29)	8 (28)
Fall Creek	0 (23)	0 (26)	0 (26)	8 (28)	7 (23)	0* (2*)	1* (11*)
Lookout Point	0 (24)	1 (27)	0 (27)	0 (28)	0 (33)	0 (30)	0 (24)

* Fall Creek Reservoir was drained prior to sampling in November and only partly refilled when sampling occurred in December leaving less habitat available to sample in those months.

We focused on 48-hour deployments because it increases trap captures and allows for us to set strings at multiple sites during our sampling window. Captures are thus possible throughout the day and night periods. Even so, we would expect most captures to happen at night, when the lights provide a greater difference in illumination from background natural levels. We did not focus on new moon periods, though moonlight could modify capture efficiency (Hickford and Schiel 1999). Light intensity is also likely important and we use bright lights because other species of parasitic copepod are more attracted with increasing intensity (Novales Flamarique et al. 2009).

Inexpensive traps can be important to achieve research goals with limited funding. We expect these traps to be especially useful for rare or poorly-understood species where large-scale deployment may be necessary. They are also ideal for areas with boat traffic where traps may be stolen or damaged. The option to deploy traps at depth may provide greater captures, as other studies have found increased catches near benthic areas (Tranter et al. 1981; Tor et al. 2009). Their small size can make plankton easy to damage by netting and pumping (Beers et al. 1967); damage is less likely with light trapping methods, especially using rigid traps, because taxa are not forced against a net. Inexpensive lights that function below 30 m are likely to be useful in an even broader context, as additional sampling and surveying methods rely on light for attraction or visibility. While here we use 445–450 nm wavelength LEDs, a wide variety of options are available. Spectral sensitivity for *S. californiensis* at 445–450 nm would not be unexpected given that other freshwater taxa have shown similar maxima (Forward 1988). Longer wavelengths (550 nm) have been documented as the maximum sensitivity for marine parasitic copepodids (Bron 1993) and peak sensitivity to 475–525 nm wavelengths is common for taxa in estuarine, coastal and open ocean habitats, though some taxa may have peak sensitivity as low as 360 nm (Forward 1988).

Future studies could explore the efficiency of these traps, especially for other taxa and the changes in capture composition with alternative LEDs and/or differing LED intensities. Blue and green wavelengths are likely ideal for a broad range of taxa and transmit well in water (McLeod and Costello 2017). Using a microcontroller, while not pursued in our final design, would allow for lights in series, patterns in illumination and the fine tuning of light intensity. Shorter duration deployments could explore day versus night efficiencies and may allow for the use of smaller batteries and possible further downscaling of the design presented here.

Outside of light trapping, we expect that the instructions (Supplement) for underwater light construction and rubber weights may be broadly applicable. Our description of how to assemble LED light sources for use underwater allows for researchers to customise wavelengths and target desired taxa. Low-cost selective wavelength LEDs may also be useful for other underwater applications (e.g. video). Weights that use discarded materials (bike tubes) and sand can aid in balancing equipment underwater where cost or vandalism are of concern. In our case, the overall affordability of the trap and components allows for the extensive trapping needed to capture and map the occurrence of rarely-observed species and lifestages, such as the copepodids of *S. californiensis*. In general, increasing the number of traps that can be deployed within or across sites can aid in the spatial comparisons of plankton distributions needed in studies of ecology and life history. Effective trapping of zooplankton, parasitic and free-living, may be critical to early detection of invasions or novel ecological dynamics.

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Supplementary material 1

Supplementary material for a low-cost, durable, submersible light trap and customizable LED design for pelagic deployment and capture of fish parasite *Salmincola* sp. copepodids

Authors: Christina A. Murphy, William Gerth, Travis Neal, Ivan Arismendi

Data type: Pdf file.

Explanation note: **Table S1.** List of materials used to construct light traps and deployment strings. **Table S2.** Laboratory captures in light trap development. **Table S3.** Additional materials used for microcontroller programmed lights in series. **Table S4.** Examples of plankton captures categorised as Cladocera, Calanoida, Cyclopoida and *Salmincola californiensis* for Fall Creek and Cougar Reservoirs in late October 2019. **Figure S1.** Wiring diagrams. **Figure S2.** Prototype light configuration with Arduino and firmware code (.ino) for varying the light intensity in series. **Manual S1.** Pictographic light trap assembly instructions.

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RESEARCH ARTICLE

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Competitive effects of plant invaders on and their responses to native species assemblages change over time

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Abstract

Alien plant invaders are often considered to be more competitive than natives, and species-rich plant communities are often considered to be more resistant to invaders than species-poor communities. However, the competitive interactions between invaders and assemblages of different species richness are unlikely to be static over time (e.g. during a growth season). To test this, we grew five alien and five native species as invaders in a total of 21 artificial assemblages of one, two or four native competitor species. To test for temporal changes in the reciprocal effects of invaders and the competitor assemblages on each other, and how these depend on the species richness of the assemblages, we harvested plants at three growth stages (weeks 4, 8 and 12). We found that the invaders and competitor assemblages had negative effects on each other. Aboveground biomass of invaders was reduced by the presence of a competitor assemblage, irrespective of its species richness, and this difference gradually increased over time. Alien invaders accumulated more aboveground biomass than the native invaders, but only after 12 weeks of growth. Meanwhile, the invaders also negatively affected the biomass of the competitor assemblages. For multi-species assemblages, the increase in the negative effect of the presence of the invader occurred mainly between weeks 4 and 8, whereas it happened mainly between weeks 8 and 12 for the one-species assemblages. Our results suggest that although alien invaders are more competitive than native invaders, the competitive effects of the invaders on and their responses to native competitor assemblages changed over time, irrespective of the origin of the invaders.

Keywords

Coexistence, community assembly, diversity-invasibility, exotic, native invader, plant invasion, resistance, species richness

Introduction

Biological invasions, as one of the major components of global change (Hobbs and Mooney 2005; Vilà et al. 2011; Essl et al. 2020), have become a matter of great concern in recent decades. More and more alien species have established populations outside their native ranges (Strayer 2010; Dawson et al. 2017), resulting in biotic homogenization (Yang et al. 2021). The accumulation of such naturalized alien species is still increasing globally and forecasted to continue increasing (Seebens et al. 2017; Seebens et al. 2021). Some naturalized alien plant species have become widespread and dominant, and are considered invasive as they seriously threaten native biodiversity and ecosystem functioning (Richardson et al. 2000; Vilà et al. 2011). The factors allowing alien species to establish, their impacts on the native species they compete with, and what allows communities to resist invaders have therefore become major research questions in ecology.

Although invasion biology focuses on alien species, the process of invasion is not restricted to alien species, as native species can also invade communities (Valéry et al. 2008, 2009; Carey et al. 2012). Actually, invasion (or colonization) by native species is an inherent part of community assembly and metacommunity dynamics, and invading native species can also impact the other species (Holyoak et al. 2005). However, as alien and native species differ in their eco-evolutionary experience with the other community members, their invasion dynamics might differ (Saul et al. 2013). Therefore, a major question is whether the establishment and impacts of alien species differ from those of native species.

The relationships between invaders and the species they interact with has been of high interest to biologists for a long time (Darwin 1859; Elton 1958; Fridley et al. 2007; Howeth 2017; Li et al. 2021). For example, Elton (1958) proposed that more diverse communities should be more resistant to invaders. This now classic diversity-invasibility hypothesis is based on the idea that more niches are already occupied in species-rich communities than in species-poor ones. This reduces the available resources in species-rich communities and thereby creates a more competitive environment. Consequently, it will be more difficult for invaders to establish when they have to compete with multiple species (Knops et al. 1999; Levine et al. 2004). While theoretical studies generally support the diversity-invasibility hypothesis, empirical studies have provided inconsistent results (Levine and D'Antonio 1999). In particular, studies at large spatial scales frequently find positive instead of negative relationships (Levine 2000; Shea and Chesson 2002). However, even in studies at small spatial scales, the diversity-invasibility relationship is not always negative, as it can depend on the environmental conditions (Naeem et al. 2000; Zeiter and Stampfli 2012) and community productivity (Davies et al. 2007). In more diverse communities, the growth of plants can be limited by resource availability (e.g. light and nutrients), and this may also affect the possibility of invasion (Mata et al. 2013; Kelso et al. 2020).

While invaders may impact the native community, and the latter might affect the establishment success of the invader, these competitive effects and responses are not static over time (Dostál et al. 2013; Yelenik and D'Antonio 2013). Some studies have shown that the negative effects of invaders on their competitors are more pronounced at the early stages, that is, the superior competitiveness of invaders is more likely to provide advantages in the early stages of growth (Goldberg 1990; Golivets and Wallin 2018). Meanwhile, the change in reciprocal effects may also be related to the species richness of the community (i.e. the competitive environment; Clark and Johnston 2011; Clark et al. 2013). Studies have shown that species-richness effects can become important during later stages of establishment (Roscher et al. 2013; Nitschke et al. 2010). After a period of growth, a species-rich community can establish a more stable community structure (Cavieres and Badano 2009), so that invaders are more strongly suppressed than when competing in a species-poor community. However, it could also be that the often high competitive ability of alien invaders may be sufficient to overcome the competitive pressures (Ridenour et al. 2008; Golivets and Wallin 2018). In that case, the biomass of the competitors could decrease without obvious suppression of the invaders. Therefore, the competitive effects and responses of invaders need to be assessed at different time points during the growth period.

To test how alien and native plant invaders and native competitor assemblages of different species richness affect each other over time, we conducted a mesocosm experiment using five alien and five native invader species and 21 competitor assemblages of three species-richness levels (1, 2 or 4 native species). To test if competitive effects and responses of invaders changed over time, we had three harvesting times (4, 8 and 12 weeks after the start of the experiment). We addressed the following specific questions: (1) How does the presence of a competitor assemblage (i.e. a community) affect growth of the invader, and does it depend on the origin of the invader and species richness of the competitor assemblage? (2) How do invaders affect the productivity of the plants they compete with, and does it depend on the origin of the invader and species richness of the competitor assemblage? (3) Do the competitive effects and responses of the invader change over time?

Materials and methods

Study species

To test the effects of alien and native invaders on competitor assemblages of different diversities, we selected five pairs of taxonomically related species to be used as invaders. Each pair consisted of one species that is a naturalized alien and one species that is native to Germany. The five pairs of species are from four families, as we chose two pairs of Poaceae so that the numbers of grasses and forbs were relatively balanced (Suppl. material 1: Table S1). To test the effects of competitor assemblages of different diversities on the invaders, we chose a native species pool of seven species (five grasses and two forbs) that frequently coexist in German grasslands (Suppl. material 1: Table S2A). The alien-native classification of all species used in the experiment was based on the FloraWeb database (www.floraweb.de). Seeds of six species were from the Botanical Garden of the University of Konstanz, and seeds of the other 11 species were ordered from Rieger-Hofmann GmbH (Suppl. material 1: Tables S1, S2A).

Experimental set up

From 10 to 17 February 2020, we sowed the invader and competitor species in trays (18 cm × 14 cm × 5 cm) filled with potting soil (Einheitserde, Gebr. Patzer GmbH & Co. KG, Sinntal, Germany). This was done on different dates (Suppl. material 1: Tables S1, S2A), based on prior knowledge about the time required for germination, so that the seedlings would be in a similar growth stage at transplanting. Seedling cultivation was done in a greenhouse of the Botanical Garden of the University of Konstanz (47°41'33"N, 9°10'35"E) with a temperature maintained between 18 °C and 25 °C.

For the experiment, we filled 3L pots (Φ = 16 cm, H = 12 cm) with a soil substrate consisting of a mixture of field soil, sand and vermiculite (v:v:v = 1:1.5:1.5). The field soil, which served as inoculum of a natural soil microbiome, was dug up from a native grassland patch in the Botanical Garden of the University of Konstanz and was sieved using a 1-cm metal mesh to remove large plant fragments and pebbles. On 3 and 4 March 2020, we transplanted the seedlings into the pots. We used the pool of seven native species to create a total of 21 competitor assemblages that had different species-richness levels (Suppl. material 1: Table S2B): one, two and four species. For each of the three species-richness levels, we had seven different assemblages. We first transplanted into each pot four individuals of the competitor assemblage. Specifically, we planted four seedlings of the same species in the one-species pots, two seedlings from each of the two species in the two-species pots, and one seedling from each of the four species in the four-species pots. After planting the competitors, we transplanted one of the ten invaders in the center of each pot (Fig. 1). To assess the effect of the presence of a competitor assemblage on the invader, we also had pots in which we only planted a single invader individual (i.e. without competitors; zero-species assemblage). Furthermore, to assess the effect of the invader on the competitors, we also had pots for each competitor assemblage in which we only planted the four individuals of the competitors (i.e. without an invader). Seedlings that died within two weeks after transplanting were replaced. For the two control treatments (i.e. only with invader, only with competitors), we replicated each invader species or competitor assemblage three times, resulting in 909 pots in total ([21 assemblages × 10 invaders + 21 assemblages × 3 replicates + 10 invaders × 3 replicates] × 3 harvest times). All pots were placed on plastic dishes (Φ = 20 cm) and randomly allocated to positions in three greenhouse compartments (24 °C/18 °C day/night temperature, 16h/8h day/night light). We watered the plants every 1–2 days, and fertilized them six times (1, 3, 5, 7, 9 and 11 weeks after the start of the experiment) with a water-soluble fertilizer (1‰ m/v, Universol Blue).

Measurements

At the start of the experiment, we counted on each invader seedling the number of leaves, and measured the length and width of the largest leaf. From these measurements, we calculated the initial leaf area as number of leaves × length of largest leaf × width

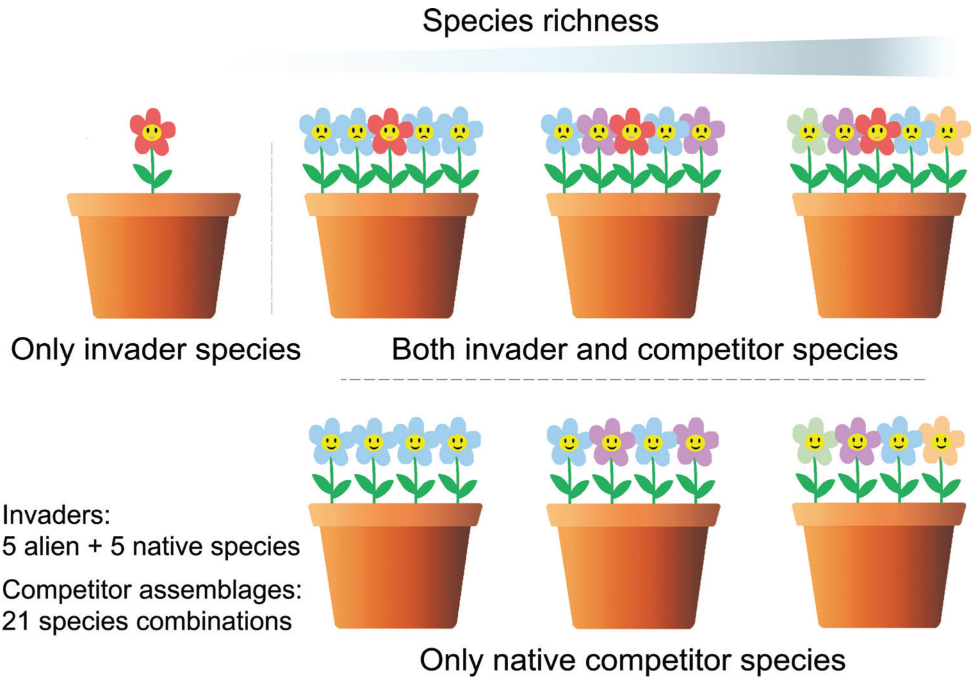

Figure 1. Overview of the experimental design. For the treatments with both an invader and competitors, five seedlings (one invader and four competitor seedlings) were planted into each pot. There was one control treatment with only plants of the native competitor assemblage (four seedlings per pot). The competitor assemblages were created with three species-richness levels: one, two and four native species. Another control treatment had only the invader species (without the competitors; one seedling per pot). All treatment combinations were replicated three times, and one replicate was harvested at each of the three time points (weeks 4, 8 and 12).

of largest leaf. In order to test the reciprocal effects of invaders and competitors over time, we selected three time points for harvesting: week 4, week 8 and week 12. These time points were chosen to represent the early, mid and late growth stages of the species during a season. On 15 April, 13 May and 10 June 2020, we harvested one third of the plants in each treatment combination. After each harvest, the remaining pots were re-randomized to reduce potential effects of environmental heterogeneity in the greenhouse compartments. We separately harvested the aboveground biomass of each individual plant. The belowground biomass, we only harvested at week 4, because it was impossible to separate the roots of the different species at weeks 8 and 12. The biomass of each individual was dried to constant weight at 70 °C, and then weighed with an accuracy of 0.001g. To compare differences in biomass between treatments, we calculated the percentage of change in biomass, using the raw data, as (Mean of biomass in the focal treatment - Mean of biomass in reference treatment)/Mean of biomass in reference treatment.

Statistical analysis

To test the effects of origin of the invader and species richness of the competitor assemblage on invader performance over time, we fitted a linear mixed model with the *lme* function in the R package 'nlme' (Pinheiro et al. 2019). This was done for the subset of pots with invaders, and aboveground biomass of the invaders was the main response variable. In addition, to test whether the dominance of the invader relative to the competitors depended on origin of the invader, species richness of the competitor assemblage and time, we also analyzed the proportional invader biomass (i.e. aboveground biomass of the invader / [aboveground biomass of the invader + aboveground biomass of the competitors]) as the response variable. The latter was done for the subset of pots with both invader and competitor plants. Invader origin (alien or native), species richness of the competitor assemblage (0, 1, 2 and 4 species or 1, 2 and 4 species when pots without competitors were excluded), harvesting time (weeks 4, 8 and 12) and their interactions were included as fixed effects in the models. For species richness of the competitor assemblage, we also ran orthogonal hierarchical contrasts to test the effect of the presence of the competitors (i.e. without competitors vs. the average of one-, two- and four-species competitor assemblages; this contrast was not included for proportional aboveground biomass), the effect of having multiple species as competitors (i.e. one-species assemblages vs. the average of two- and four-species assemblages), and the effect of having more species in the multi-species competitor assemblages (i.e. two-species assemblages vs. four-species assemblages). To account for variation in initial size of the invaders, we included the initial leaf area of the invaders as a covariate.

To test the effects of the presence of the invader and its origin, and of the species richness of the competitor assemblages on performance of the assemblages over time, we fitted again a linear mixed model. This was done for the subset of pots with competitors, and aboveground biomass of the competitor assemblage and total aboveground biomass per pot (i.e. cumulative biomass of the invader and competitors) were used as response variables. Invader treatment (without invader, with alien invader or with native invader), species richness of the competitor assemblage (1, 2 and 4 species), harvesting time (weeks 4, 8 and 12) and their interactions were included as fixed effects. For the invader treatment, we generated two orthogonal contrasts: without vs. with invader, and alien vs. native invader. We also generated two orthogonal contrasts for species richness of the competitor assemblage: one-species assemblages vs. the average of two- and four-species assemblages, and two-species assemblages vs. four-species assemblages.

To test whether the belowground parts of plants show a similar response as the aboveground parts, we also fitted two linear mixed effects models to analyze the belowground biomass of the invaders and competitor assemblages, respectively, at the first harvest time (i.e. week 4). For the invaders, this was done for the subset of pots with invaders in week 4, and belowground biomass and root weight ratio of the invaders were the response variables. Invader origin (alien or native), species richness of the competitor assemblages (included as three orthogonal contrasts: without competitors vs. the average of one-, two- and four-species assemblages, one-species assemblages vs. the average of two- and four-species assemblages, and two-species assemblages vs.

four-species assemblages) and their interactions were included as fixed effects. We also included initial leaf area of the invaders as a covariate in the model. For the competitor assemblages, belowground biomass and root weight ratio (i.e. belowground biomass allocation) for the subset of pots with competitors in week 4 were used as response variables. Invader treatment (two orthogonal contrasts: without vs. with invader, and alien vs. native invader), species richness of the competitor assemblage (included as two orthogonal contrasts: one-species assemblages vs. the average of two- and four-species assemblages, and two-species assemblages vs. four species assemblages) and their interactions were included as fixed effects.

In all models, to account for phylogenetic non-independence of species, and non-independence of plants belonging to the same species, we included species identity and family of the invader plants as random effects. To account for non-independence of measurements in pots with the same competitor assemblage, we also included assemblage identity as a random effect. To meet the assumption of normality, aboveground biomasses of invaders and competitor assemblages were cubic-root-transformed. To improve homoscedasticity of residuals of the models, we allowed the variance to vary among invader species and/or the assemblage identity (Suppl. material 1: Table S6) by using the *varComb* and/or *varIdent* functions of 'nlme' package. For all models, we used log-likelihood ratios, which are approximately χ^2 distributed, to assess the significances of the fixed effects by comparing models with and without the effect of interest (Zuur et al. 2009). All analyses were conducted with R 3.6.2 (R Core Team 2019). An effect was considered significant if $P < 0.05$.

Results

Effects of competitor presence and species richness on invaders at different times

Across all competitor-assemblage treatments, the aboveground biomass of alien and native invaders did not differ significantly after four and eight weeks of growth (Table 1, Fig. 2A). However, after 12 weeks of growth, the alien invaders had produced significantly more aboveground biomass than the native ones (+16.3%; Table 1, Fig. 2A). Compared to the treatment without competitors (i.e. 0-species assemblage), aboveground biomass of invaders was significantly lower in the presence of competitors, and this difference gradually increased over time (-35.5% in week 4, -53.1% in week 8, and -55.5% in week 12; Table 1, Fig. 2B). Belowground biomass of the invader, which was only measured at week 4, was also significantly reduced by the presence of competitors (-45.3%), while the root weight ratio was not significantly affected (Suppl. material 1: Table S4, Fig. S1).

Among the pots with competitors, aboveground biomass of the invader was not significantly affected by the species richness of the competitor assemblage (one-species vs. multi-species assemblages, and two-species vs. four-species assemblages; Table 1, Fig. 2B). The proportional biomass of the invaders relative to the competitors was not significantly affected by the origin of the invader, species richness of the competitor assemblage and time (Fig. 3C, Suppl. material 1: Table S3).

Table 1. Effects of invader origins (alien or native), presence and species richness of the native competitor assemblage (0, 1, 2 or 4 species), harvesting time (week 4, week 8 or week 12) and their interactions on aboveground biomass of invader plants. For the factor Species richness, we created three orthogonal contrasts ($R_{\text{Without/With}}$: without competitors vs. average of one-, two- and four species assemblages, $R_{\text{One-/Multi-species}}$: one-species assemblages vs. average of two- and four-species assemblages, $R_{\text{Two-/Four-species}}$: two-species assemblages vs. four-species assemblages).

	df	**Aboveground biomass**	
		χ^2	*P*
Fixed effects			
Initial leaf area of invader	1	15.542	**<0.001**
Origin of invader (O)	1	0.339	0.560
Species richness of assemblage (R)	3	18.319	**<0.001**
$R_{\text{Without/With}}$	1	17.643	**<0.001**
$R_{\text{One-/Multi-species}}$	1	0.750	0.387
$R_{\text{Two-/Four-species}}$	1	0.868	0.352
Time of harvest (T)	2	479.275	**<0.001**
O × R	3	0.950	0.813
O × $R_{\text{Without/With}}$	1	0.179	0.672
O × $R_{\text{One-/Multi-species}}$	1	0.780	0.377
O × $R_{\text{Two-/Four-species}}$	1	0.006	0.936
O × T	2	8.655	**0.013**
R × T	6	83.415	**<0.001**
$R_{\text{Without/With}}$ × T	2	79.256	**<0.001**
$R_{\text{One-/Multi-species}}$ × T	2	4.435	0.109
$R_{\text{Two-/Four-species}}$ × T	2	0.614	0.736
O × R× T	6	1.921	0.927
O × $R_{\text{Without/With}}$ × T	2	0.492	0.782
O × $R_{\text{One-/Multi-species}}$ × T	2	1.034	0.596
O × $R_{\text{Two-/Four-species}}$ × T	2	0.380	0.827
Random effects		SD	
Invader family		0.206	
Invader species [†]		0.314	
Assemblage identity		0.068	
Residual		0.166	

Values are in bold when $P < 0.05$. [†] Shown is the standard deviation (SD) of *Lepidium virginicum*. The SDs of all invader species are shown in Suppl. material 1: Table S6.

Effects of invaders on competitors and overall productivity at different times

After four weeks of growth, the aboveground biomass of the competitor assemblage, irrespective of its species richness, was not affected by the presence of the invader. The same was true for belowground biomass and the root weight ratio of the competitor assemblages (Suppl. material 1: Table S5, Fig. S2). After 8 and 12 weeks of growth, however, the presence of the invader had a significant negative effect on the aboveground biomass of the competitors (Table 2, Fig. 3A). Moreover, while the increase in the negative effect of the presence of the invader happened mainly between weeks 4 and 8 for the multi-species competitor assemblages (two-species assemblages: -8.4% in week

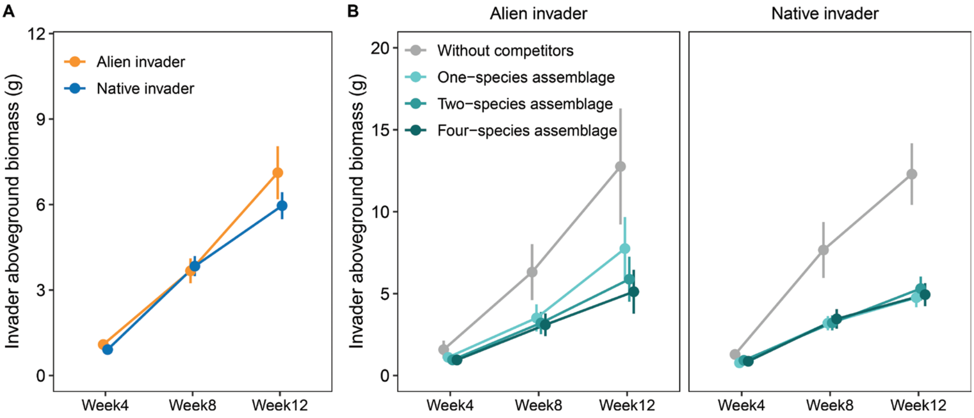

Figure 2. A aboveground biomass of alien and native invaders at each of the three harvests, **B** aboveground biomass of alien and native invaders in the absence or presence of native competitor assemblages of different species richness at each of the three harvests. Shown are means (± SEs) of the raw data.

4, -28.7% in week 8 and -15.7% in week 12; four-species assemblages: -0.2% in week 4, -20.6% in week 8 and -11.0% in week 12), it happened mainly between weeks 8 and 12 for the one-species assemblages (-2.7% in week 4, -12.5% in week 8 and -27.1% in week 12; Table 2, Fig. 3A). The origin of the invader did not have a significant effect on the aboveground biomass of the competitor assemblage (Table 2, Fig. 3A).

The total aboveground biomass per pot was not significantly affected by the species richness of the competitor assemblage (Table 2, Fig. 3B). However, having an invader plant, in addition to the competitors, increased biomass for the one-species assemblages after week 4, whereas, for the multi-species assemblages, it only increased biomass at week 12 (significant $I_{\text{Without/With}} \times R_{\text{One-/Multi-species}} \times T$ in Table 2, Fig. 3B). There was no significant effect of the origin of the invader (Table 2, Fig. 3B).

Discussion

In our experiment on competitive effects and responses of native and alien invaders over time, we found that the invaders had strongly reduced biomass in the presence of the competitors. This negative effect of the competitors on the invaders strongly increased during the growth period, but did not significantly depend on the species richness of the competitor assemblage. The alien and native invaders produced similar amounts of biomass during the first eight weeks, but after 12 weeks, the alien invaders had produced more biomass than the native ones. Similarly, addition of single invader plants also suppressed the biomass production of the competitor assemblage, and this effect also increased over time. In the multi-species competitor assemblages (two- and four-species assemblages), this effect was already pronounced after eight weeks, whereas in the one-species assemblages, it became most obvious after twelve weeks and then

Table 2. Effects of invader treatment (without invader, with alien or native invader), species richness of competitor assemblage (1, 2 or 4 species), harvesting time (week 4, week 8 or week 12) and their interactions on aboveground biomass of the competitor assemblage and the total aboveground biomass per pot. For the factor Invader, we created two orthogonal contrasts ($I_{\text{Without/With}}$: without vs. with invader, $I_{\text{Alien/Native}}$: with alien vs. with native invader). For the factor Species richness, we created two orthogonal contrasts ($R_{\text{One-/Multi-species}}$: one-species assemblages vs. average of two- and four species assemblages, $R_{\text{Two-/Four-species}}$: two-species assemblages vs. four-species assemblages).

	df	**Aboveground biomass of competitors**		**Total aboveground biomass per pot**	
		χ^2	*P*	χ^2	*P*
Fixed effects					
Invader treatment (I)	2	2.870	0.238	0.505	0.777
$I_{\text{Without/With}}$	1	2.559	0.110	0.505	0.477
$I_{\text{Alien/Native}}$	1	0.368	0.544	0.000	0.997
Species richness of assemblage (R)	2	1.507	0.471	1.351	0.509
$R_{\text{One-/Multi-species}}$	1	1.506	0.220	1.011	0.315
$R_{\text{Two-/Four-species}}$	1	0.001	0.978	0.368	0.544
Time of harvest (T)	2	1877.817	**<0.001**	1888.372	**<0.001**
I × R	4	7.421	0.115	1.860	0.761
$I_{\text{Without/With}} \times R_{\text{One-/Multi-species}}$	1	0.003	0.954	0.020	0.889
$I_{\text{Without/With}} \times R_{\text{Two-/Four-species}}$	1	5.053	**0.025**	0.842	0.359
$I_{\text{Alien/Native}} \times R_{\text{One-/Multi-species}}$	1	0.006	0.938	1.041	0.308
$I_{\text{Alien/Native}} \times R_{\text{Two-/Four-species}}$	1	2.375	0.123	0.032	0.858
I × T	4	29.829	**<0.001**	5.454	0.244
$I_{\text{Without/With}} \times T$	2	29.675	**<0.001**	4.026	0.134
$I_{\text{Alien/Native}} \times T$	2	0.197	0.906	1.534	0.464
R × T	4	5.760	0.218	0.874	0.928
$R_{\text{One-/Multi-species}} \times T$	2	0.068	0.967	0.103	0.950
$R_{\text{Two-/Four-species}} \times T$	2	5.593	0.061	0.795	0.672
I × R × T	8	14.863	0.062	11.242	0.188
$I_{\text{Without/With}} \times R_{\text{One-/Multi-species}} \times T$	2	11.933	**0.003**	8.667	**0.013**
$I_{\text{Without/With}} \times R_{\text{Two-/Four-species}} \times T$	2	0.885	0.643	0.491	0.782
$I_{\text{Alien/Native}} \times R_{\text{One-/Multi-species}} \times T$	2	0.049	0.976	0.650	0.723
$I_{\text{Alien/Native}} \times R_{\text{Two-/Four-species}} \times T$	2	2.115	0.347	1.529	0.466
Random effects		SD		SD	
Invader family		0.001		0.073	
Invader species		0.056		0.141	
Assemblage identity†		0.083		0.048	
Residual		0.099		0.105	

Values are in bold when $P < 0.05$. † Shown is the standard deviation (SD) of the one-species assemblage of *Lolium perenne*. The SDs of all assemblage identities are shown in Suppl. material 1: Table S6.

even more pronounced than in the multi-species assemblages. So, although our results did not indicate major roles of the origin of the invader and the species richness of the competitor assemblage, we found that the invader and competitors reciprocally suppressed one another, and that these interactions became more intense over time.

The alien invaders only produced more biomass than the native invaders by week 12. As invasive alien plants are frequently characterized by fast early growth (Grotkopp et al. 2010; Dawson et al. 2011), and frequently produce more biomass than native species (van Kleunen et al. 2010), it is surprising that the difference in biomass did not appear earlier. It could be that the delay is due to the resistance provided by the competitors, which slowed down the overall growth rates of the invaders. Meanwhile, all the seedlings per pot were transplanted at the beginning of the experiment, which may eliminate the effect of differences in the phenological niche between the competitor species, and between the competitor assemblage and the invader in the growth stages (Wolkovich and Cleland 2011; Godoy and Levine 2014). Furthermore, it could reflect that not all naturalized alien species in our study are highly invasive and that the native invaders themselves are also very common. The fact that the alien invaders nevertheless produced more biomass than the native invaders at the end of the experiment may reflect that many naturalized alien species are more competitive than natives (Vilà and Weiner 2004; Kuebbing and Nuñez 2016; Golivets and Wallin 2018; Zhang and van Kleunen 2019). Another reason why alien invaders ultimately performed better than native invaders may be that they have escaped from the co-evolved enemies in their native ranges (Keane and Crawley 2002). As we did our experiment in a greenhouse environment, it is likely that both the aliens and natives were released from aboveground herbivores. However, as we provided all pots with an inoculum of field soil, it is likely that the soil contained root herbivores and pathogens that might have preferentially attacked the native plants. The differences in competitive ability among the native plants can have been equalized by the native soil pathogens (Albornoz et al. 2017). Despite the difference in biomass between the alien and native invaders at the end of the experiment, they did not have different effects on the biomass production of the competitor assemblages. Moreover, the proportional biomass of the alien and native invaders in the presence of competitors was not significantly different (Fig. 3C). In other words, the slight superiority in biomass production of the alien invaders did not result in a larger dominance, at least not during the 12 weeks of our experiment.

While the presence of competitors significantly reduced the biomass of the invader, the effect of species richness of the competitor assemblage was not significant. In other words, we did not find support for Elton (1958)'s diversity-invasibility hypothesis. Other studies found that invader species could be strongly limited by nutrient and light availability when they competed with more diverse species assemblages (Roscher et al. 2009; Mata et al. 2013), whereas other studies found also no significant relationship (Smith and Côté 2019) or actually found a positive one (Jiang and Morin 2004; Zeiter and Stampfli 2012). It has, however, also been reported that the diversity-invasibility relationship can change with time (Clark and Johnston 2011). Actually, at week 12 (i.e. at the end of our experiment), there was a slight, though non-significant, trend that the invader aboveground biomass was lowest in four-species competitor assemblages and highest in one-species assemblages (Fig. 2B). So, possibly if the experiment would have lasted longer, the effect might have become significant. This would be particularly likely, if complementarity effects increase with time (Fargione et al. 2007). It could also be that diversity effects on resistance against invaders require higher species-richness levels than we used. Therefore, we recommend that future invasion experiments use

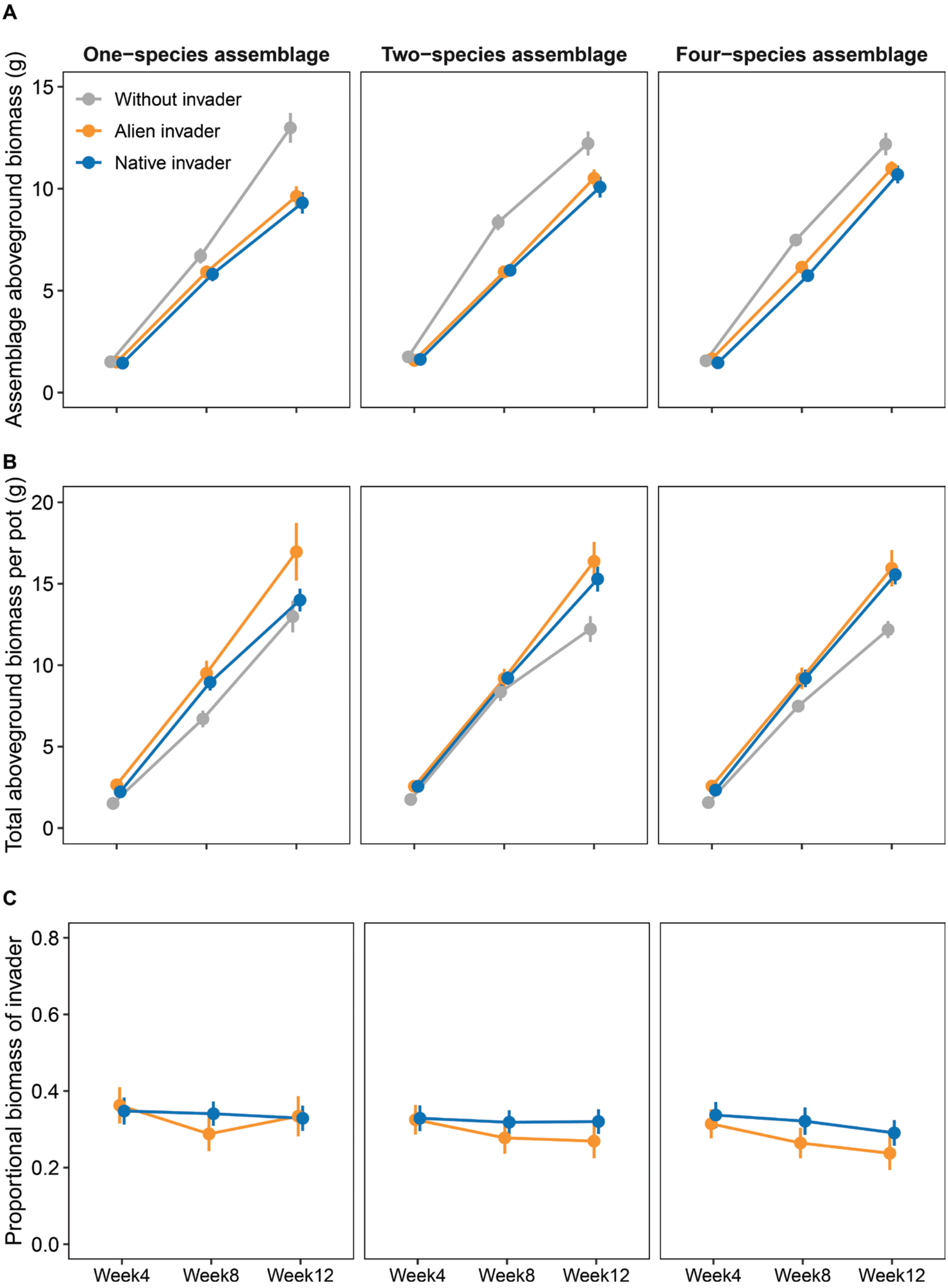

Figure 3. Aboveground biomass of native competitor assemblages (**A**) and total aboveground biomass per pot (**B**) for different competitor assemblages of different species richness and in the absence or presence of alien and native invaders at each of the three harvests, **C** proportional aboveground biomass per pot of alien and native invaders in competitor assemblages of different species richness at each of the three harvests. Shown are means (± SEs) of the raw data.

species assemblages that are older and have higher maximum species-richness values than the ones we used.

A potential limitation of our study is that the species pool that we used to create the competitor assemblages was relatively small (n = 7). As a consequence, some of the two-species assemblages shared species with one another, and this sharing was even stronger for the four-species assemblages (each species was present in 4 of the 7 four-species assemblages). In other words, with increasing species richness, the assemblages became more similar to each other. In our study this confounding most likely had no major consequences as there were no significant effects of species richness. Visual inspection of the biomass development of each of the competitor assemblages (Suppl. material 1: Fig. S3) also did not reveal clear indications that the presence of a particular species drove the differences in biomass among the assemblages. Nevertheless, we recommend that future experimental studies on the diversity-invasibility hypothesis use larger species pools to avoid such effects.

Like the presence of competitors reduced the biomass of the invader, so did reciprocally the presence of the invader reduce the biomass of the competitors. This most likely reflects that the addition of the invader increased the density of plants per pot, and that this resulted in more intense competition among the plants (Callaway and Walker 1997; Zhang and Tielbörger 2020). Moreover, the effect of the invader on the biomass of the competitor assemblage increased over time, but the pattern of this increase depended on whether the assemblage consisted of one or multiple species (Fig. 3A). Surprisingly, although we had expected that already early on in the experiment the one-species competitor assemblages would suffer more from the invader than the multi-species assemblages, the one-species assemblages only showed a clear effect of the invader at week 12, whereas the multi-species assemblages showed it already at week 8. Possibly, early on, at week 4, the plants were still so small that they hardly interacted with one another. At week 8, the plants in the one-species assemblages without invaders were still relatively small, while in the multi-species assemblages the plants were larger, as intraspecific competition is usually more intense than interspecific competition (Adler et al. 2018). Consequently, in the presence of the invader, the one-species competitor assemblages still hardly interacted with the invader, whereas the multi-species assemblages had to share their resources with the invader, and therefore produced less biomass. Indeed, the joint invader and competitor biomass at week 8 differed less between the invaded and non-invaded multi-species assemblages than between the invaded and non-invaded one-species assemblages (Fig. 3B). Whatever the exact reason is for these different patterns over time, at the last census the negative effect of the invader was, as expected, larger for the one-species competitor assemblages than for the multi-species ones. Again this effect might have become even stronger if the experiment would have lasted longer.

In conclusion, we found reciprocal effects of invaders and competitors, and that these effects became stronger over time. Although the alien invaders produced more biomass than the native invaders by the end of the experiment, they were not yet differently affected by the presence and species richness of the competitor assemblages. The effects of the invader on the competitors also did not yet depend on whether the invader was an alien or a native. However, at the end of the experiment, the one-species competitor assemblages were more strongly affected by the invader than the multi-species ones. So, even though our results did not support the diversity-invasibility hypothesis, if the effects that we found continue to increase over time the hypothesis might hold.

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Supplementary material 1

Table S1–S6, Figs S1–S3

Authors: Duo Chen, Mark van Kleunen

Data type: PDF file

Explanation note: **Table S1.** Alien and native invader species used in the experiment. **Table S2.** Competitor species used in the experiment and combinations of species to produce seven native competitor assemblages for each of the three species-richness levels. **Table S3.** Effects of invader origins, species richness of the competitor assemblage, harvesting time and their interactions on proportional aboveground biomass of invader plants. **Table S4.** Effects of invader origins, presence and species richness of the competitor assemblage and their interactions on belowground biomass and root weight ratio of invader plants at the first harvest time. **Table S5.** Effects of invader types treatment, species richness of competitor assemblage and their interactions on belowground biomass and root weight ratio of the native competitor assemblage at the first harvest time. **Table S6.** The SDs of the ten invader species and/or 21 assemblage identities from the models shown in Tables 1, 2, S3, S4 and S5. **Fig. S1.** Belowground biomass and root weight ratio of invaders in the absence or presence of native competitor assemblages of different species richness at the first harvest. **Fig. S2.** Belowground biomass and root weight ratio of the competitor assemblage in the absence or presence of alien and native invader species at the first harvest. **Fig. S3.** Aboveground biomass of each of 21 native competitor-assemblage combinations in the absence or presence of alien and native invaders at each of the three harvests.

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Alien plants tend to occur in species-poor communities

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Abstract

Invasive alien species can have severe negative impacts on natural ecosystems. These impacts may be particularly pronounced within ecological communities, where alien species can cause local extinctions. However, it is unclear whether individual alien plant species consistently occur in species-poor or species-rich communities across broad geographical scales and whether this pattern differs amongst habitat types. Using ~17,000 vegetation plots sampled across the Czech Republic, we calculated the median, range and skewness of the distribution in community species richness associated with 73 naturalised alien plant species. We compared the observed values with those obtained under a null expectation to test whether alien species occurred at random with respect to species richness in forest and grassland communities. We found that the relationship between the occurrence of alien species and the diversity of local plant communities was species-dependent and varied across habitats. Overall, however, alien species occurred in species-poor communities more often than expected by chance. These patterns were more pronounced in grasslands, where alien species also occurred in communities with a lower range of species richness than under random expectation. Our study represents one of the most comprehensive quantitative analyses relating alien plant invasion to resident community diversity at a broad geographical scale. This research also demonstrates that multi-species studies are needed to understand the processes of community assembly and to assess the impact of alien plant invasions on native diversity.

Keywords

biotic acceptance, biotic resistance, community ecology, Czech Republic, plant invasion, species richness

Introduction

The spread of alien invasive species has serious environmental and socioeconomic impacts (Vitousek et al. 1997; Richardson and Pyšek 2008). These impacts are evident across spatial scales, but can be particularly pronounced within ecological communities, where invasive species can alter species composition and lead to local extinctions (Richardson and Pyšek 2006). Identifying the mechanisms of alien plant invasion in ecological communities is crucial for understanding the factors that support and constrain biodiversity and, ultimately, for designing effective management plans.

In recent decades, many ecologists have attempted to explain what makes native communities vulnerable to invasion (Lonsdale 1999; Chytrý et al. 2008) and whether communities hosting more species are more resistant to invasion than communities with fewer species (Elton 1958; Jeschke et al. 2018). According to the “biotic resistance hypothesis”, communities with higher numbers of native species are more resistant to the establishment of incoming alien species than communities with lower numbers of native species (Elton 1958; Lonsdale 1999; Jeschke 2014). Consequently, this hypothesis predicts a negative correlation between native and alien species richness. This hypothesis is a special case of the “empty niche” hypothesis, which posits that naturalising species can occupy under-utilised niches and exploit available resources in communities unsaturated with native species (MacArthur 1970). However, empirical studies have also reported the opposite pattern, namely a positive correlation between native and alien species richness (Stohlgren et al. 1999; McKinney 2002), leading to the formulation of the “biotic acceptance hypothesis” (Stohlgren et al. 2003, 2006).

This “invasion paradox” can be partially resolved by considering the spatial scale at which biological invasions occur. Biotic resistance is thought to occur more frequently in relatively small areas where biotic interactions operate, whereas biotic acceptance is thought to become more important at larger spatial scales due to favourable environmental conditions and greater environmental heterogeneity (Levine 2000; Herben et al. 2004; Fridley et al. 2007). That is, if the environment is suitable for sustaining a high number of native species, it will also be suitable for the establishment of a high number of alien species (Naeem et al. 2000; Stohlgren et al. 2003). Nonetheless, alien species that successfully establish in species-rich communities can also reduce the diversity of recipient communities if they become dominant (Hejda et al. 2009, 2021), likely masking the net effects of biotic resistance and acceptance in observational studies. Examining the relationship between native community diversity and alien species abundance can inform us on how alien species impacts develop during the invasion process and help to design appropriate management strategies (Bradley et al. 2019).

The traditional niche theory also has difficulty explaining invasion patterns in species-rich communities because sites with high species richness generally do not provide as many niches to support such high plant diversity (Bell 2001). As an alternative, Hubbell (2001) proposed the neutral theory, which explains species coexistence within communities through stochastic processes, such as birth and death of individuals and colonisation and extinction of species. Many subsequent studies have demonstrated that deterministic and stochastic (neutral) processes are not mutually exclusive and act simultaneously to drive invasion patterns in ecological communities (Daleo et al. 2009).

Although our understanding of alien species invasion patterns has advanced significantly in recent decades, questions remain about how alien species become established in ecological communities and impact community diversity. For example, several studies have examined the association between alien species and the average species richness in the invaded communities (e.g. Fridley et al. 2007; Hejda et al. 2009; Peng et al. 2019), but no studies have yet examined the association between alien species and the range and skewness of the distribution in species richness of the invaded communities (Fig. 1). If alien species tend to occur in communities with a similar number of species, that is, over a relatively low range of species richness or short richness gradient, then this may be interpreted as a sign of a narrow niche or habitat specialisation. Within a specific range of species richness, the distribution of alien species may be asymmetric. Some species may mainly occur in species-poor communities and only establish sporadically in species-rich communities (positive skewness). In contrast, other species may mostly occur close to the higher end of the species richness gradient and only establish sporadically in species-poor communities (negative skewness). The association between alien species and community richness may also be consistent within major plant clades, which would indicate that the ecological niches of alien species are phylogenetically conserved (Wiens and Graham 2005). Combining these different parameters, which collectively define the distribution of species rich-

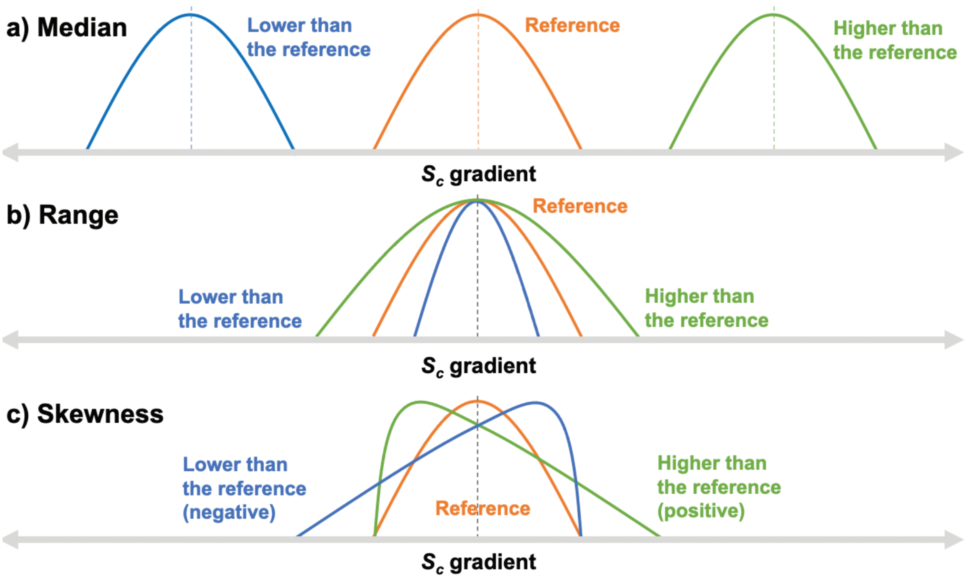


Figure 1. Schematic representation illustrating how the variation in the distribution of community species richness (S_c), associated with individual species is described by the (**a**) median, (**b**) range and (**c**) skewness.

ness associated with individual alien species, also across the tree of life, can give us insights into the ecology of alien species and improve our understanding of invasion processes.

Here, we aim to complement previous observational and empirical studies that have examined the association between the occurrence of alien species and species richness in terrestrial plant communities. We based our investigation on ~ 17,000 invaded and non-invaded vegetation plots sampled across the Czech Republic, which collectively hosted 73 naturalised alien species. Unlike previous studies, we calculated three main parameters for the tendency of individual species to occur: (1) in species-poor or species-rich communities, (2) in communities with a more or less variable number of species and (3) in communities with a symmetric or asymmetric distribution of species numbers (Fig. 1). We studied these three parameters using a null model approach to test whether the occurrence of alien species in forest and grassland communities differs from the random expectation. This knowledge can provide information for conservation plans to control the spread of aliens by targeting communities and habitats with the highest likelihood of hosting alien species.

We aim to answer two main research questions (RQs): (RQ1) Does the distribution of community species richness (median, range and skewness) associated with individual species differ between naturallzed alien and native species in forests and grasslands? (RQ2) Do naturalised alien species establish randomly in forest and grassland communities with respect to the species richness of these communities? Following the biotic resistance hypothesis, we expected that alien species would generally occur in communities with a smaller number of species than under the random expectation, regardless of the habitat in which they occur. We also expected that alien species associated with species-poor communities would generally occur in communities with a less diverse number of species and a positively skewed distribution in the number of species, indicating high specialisation of these species for stressed habitats. To complement RQ1 and RQ2 and help explain the main observed patterns, we additionally answer two secondary research questions: (RQ3) Are there consistent patterns in the relationship between community diversity and naturalised alien species amongst plant clades? (RQ4) Does the distribution of community species richness of individual naturalised alien species vary according to their level of dominance in the communities?

Methods

Vegetation data

We obtained vegetation-plot records from the Czech Republic from the Czech National Phytosociological Database (Chytrý and Rafajová 2003). Each vegetation plot in the database contains the percentage cover-abundance of all vascular plants present (in most cases derived from original data recorded using cover-abundance scales, such as the Braun-Blanquet scale). Taxon concepts and nomenclature follow the second edition of the Key to the Flora of the Czech Republic (Kaplan et al. 2019).

We assigned vegetation plots to phytosociological vegetation types (associations) following the classification system and the expert system for automatic classification developed by Chytrý (2007–2013). We excluded plots that could not be unequivocally assigned to any association from this classification. To reduce differences in sampling intensity amongst areas and vegetation types, we stratified the database by phytosociological associations and geographically (within grid cells) and randomly selected a subset of plots in the strata that contained more plots than a specified threshold (see Chytrý 2007–2013 for details). This stratified resampling resulted in 30,115 plots covering all major vegetation types in the country. We removed plots from mires, wetlands and aquatic vegetation because these habitats reflect specific environmental conditions and had a low variation in species richness. We also removed plots of unknown size from this selection. The final dataset consisted of 16,987 plots, which we grouped into two major vegetation formations dominated by either trees and shrubs or herbaceous plants and dwarf shrubs (Suppl. material 1: Appendix S1). For simplicity, we refer to these vegetation formations as "Forests" (n = 4,492) and "Grasslands" (n = 12,495; this category also includes heathlands).

We excluded all taxa of bryophytes, lichens, algae and fungi, as well as the taxa identified at the genus level. We also aggregated subspecies at the species level and some commonly misidentified groups of related species into aggregates. We applied these filters to standardise the data and remove potential biases from multiple-source sampling (e.g. bryophytes and some subspecies were not recorded in all plots). The final dataset included 1,778 species of vascular plants.

Calculation of corrected species richness (S_c)

We computed the corrected species richness (S_c) for each plot to account for variable plot size in the database (Fibich et al. 2017). This calculation was done independently for each vegetation type (i.e. forests, scrub, alpine, grasslands, rocks, screes and walls and anthropogenic vegetation) by fitting a species-area relationship (Preston 1962):

$$S = cA^z \tag{1}$$

where S is species richness (i.e. the number of vascular plant species) in the plot, A is the plot area, z is the slope of the species-area relationship in log-log space and c is a constant that depends on the unit used for area measurement and equals the number of species that would occur in a unit-sized area. We then corrected species richness to the same plot size (A_m; the median plot size in each vegetation type; Suppl. material 1: Appendix S1):

$$S_c = S(A_m/A)^z \tag{2}$$

Classification of naturalized neophytes

We classified species as "naturalised neophytes" following the national catalogue of alien species (Pyšek et al. 2012). Naturalised taxa are alien plants that reproduce in the wild and sustain populations over many life cycles without direct or despite human intervention. Neophytes are taxa occurring in the wild that humans have intentionally or unintentionally introduced to an area outside their native distribution range after the year 1500. In our classification, naturalised neophytes also included invasive neophytes. We repeated the analyses considering only "invasive neophytes" to test the robustness of our results. Invasive plants are naturalised alien plants that produce reproductive offspring, often in large numbers, at considerable distances from the parent plants, allowing them to spread over an extensive area (Richardson et al. 2000).

Statistical analyses

We performed all the analyses in R v. 4.1.0 (R Core Team 2021). To describe the distribution of community species richness associated with individual species, we assigned the S_c value of each plot to all species present in the plot. Then, we calculated the median, range and skewness S_c values of each species across all plots where it occurred (Fig. 1), separately in forests and grasslands. Finally, we calculated the mean of all median, range and skewness S_c values for all naturalised neophytes and all other species. To avoid the influence of rare species on our analyses, we did not calculate S_c values for species that occurred in fewer than five plots or fewer than 5% of plots in each vegetation formation.

The median S_c indicates the central position of the species on the species richness gradient (50th percentile). The range indicates the spread or dispersion of S_c values around the median, while skewness indicates whether S_c values are asymmetrically distributed around the median. We calculated the standardised range as the Interquartile Range (IQR = 75th percentile (Q3) – 25th percentile (Q1)) divided by the square root of the median. We standardised the range by the square root of the median because the distribution of S_c approximates a Poisson distribution and, thus, the IQR depends on the mean and median. Without standardisation, the results for the range would be governed by this mathematical relationship. The range depends linearly on the standard deviation and the standard deviation is a square root of the mean in a Poisson distribution. As for the central distribution of species richness, we also used the median, which is approximately linearly dependent on the mean.

As a measure of skewness, we calculated the Pearson moment coefficient of skewness, which is the ratio of the third central moment to the cube of the standard deviation. We then standardised this metric by subtracting the expected skewness, based on a Poisson distribution ($1/\sqrt{\text{mean}}$). After this standardisation, positive values indicate greater and negative values smaller skewness than a Poisson distribution with the same mean. We used a parametric measure of skewness because we wanted to account for the effects of outliers and extreme values in our calculations and standardised non-parametric alternatives sensitive to these were not available. Correlations between the median and standardised range and skewness of S_c can be found in Suppl. material 1: Appendix S2.

We used Mann-Whitney U Tests to identify significant differences in the distribution of observed medians, ranges and skewness of S_c between naturalised neophytes and all other species in forests and grasslands (RQ1). This analysis is efficient in displaying the distributions of the different richness parameters, but it retains the diversity gradients present in the vegetation. To remove the effects of these diversity gradients, we used a null model to test whether the mean of the median, range and skewness of S_c of naturalised neophytes differed from random expectation (RQ2). We randomised the community matrix (recoded as species presence/absence), maintaining species richness in plots and species frequency across all plots, thus without altering row and column totals. For randomisations, we used the "Curveball algorithm" (Strona et al. 2014), which can sample the set of all possible matrix configurations uniformly and requires much less computational effort than other methods, so that even large matrices can be randomised easily. Then, we recalculated the mean values of the median, range and skewness of S_c of each naturalised neophyte to obtain random S_c values for each parameter. We repeated this step 999 times to generate the null distribution of random means of the median, range and skewness of S_c. Finally, we compared the observed mean S_c of each parameter with the respective null distribution of random mean S_c and determined the *P*-values using the quantiles of the null distribution. We calculated *P*-values as the proportion of the random mean S_c that was lower than the observed mean S_c. *P*-values smaller than 0.025 indicated that the observed mean S_c of each parameter was significantly lower than expected by chance, whereas *P*-values larger than 0.975 indicated that the observed mean S_c of each parameter was significantly higher than expected by chance. We implemented the null model approach independently for each vegetation formation.

To examine whether the median, range and skewness of S_c of individual naturalised neophytes differed from the random expectation, we calculated the standardised effect sizes (SES) of these parameters as (observed parameter – mean of the expected parameter)/standard deviation of the expected parameter. For each parameter, SES < 1.96 indicates lower values than under random expectation, while SES > 1.96 indicates higher values than under random expectation. We plotted the SES of the different S_c parameters of the naturalised neophytes across the phylogeny to examine consistent patterns in ecological strategies amongst plant clades (RQ3). We created the phylogeny by linking our species to the mega-phylogeny implemented in the R package 'V.PhyloMaker' (Jin and Qian 2019). We used the 'scenario 3' approach in the same package to add missing species to the phylogeny (see more details in Jin and Qian 2019).

Finally, we examined whether the median S_c of individual neophytes varied according to their dominance in the communities (RQ4). Following Mariotte (2014), we classified species as dominant if they had relative cover ≥ 12% in each community. On the Braun-Blanquet scale, the 12% value separates species classified with the lowest degrees (i.e. r, +, 1 and 2m), which combine cover with abundance data, from those classified with the highest degrees, which are based on species cover alone. Therefore, species with relative cover ≥ 12% always have high cover and can be considered dominant in the community. We used Mann-Whitney U Tests to compare whether the distribution of median S_c differed when the species was dominant or non-dominant. We only considered species that occurred in at least ten plots in each group (i.e. dominant vs. non-dominant). We also repeated these analyses using a threshold of 25% in relative cover to test the effect of this choice on our results.

Data resources

The data underpinning the analysis reported in this paper are deposited in Zenodo at https://doi.org/10.5281/zenodo.6467402

Results

Comparing S_c of naturalised neophytes against all other species (RQ1)

We identified 25 and 60 naturalised neophytes in forests and grasslands, respectively. Compared with the other non-naturalised species, naturalised neophytes occurred more frequently in communities with fewer species in both forests (Fig. 2a) and grasslands (Fig. 2b). However, the ranges of richness values and their symmetry around the median did not differ between naturalised neophytes and all other species in either forests or grasslands. We found virtually identical results when we only considered invasive neophytes (Suppl. material 1: Appendix S3). The median and quantiles of S_c of each naturalised neophyte can be found in Suppl. material 1: Appendix S4.

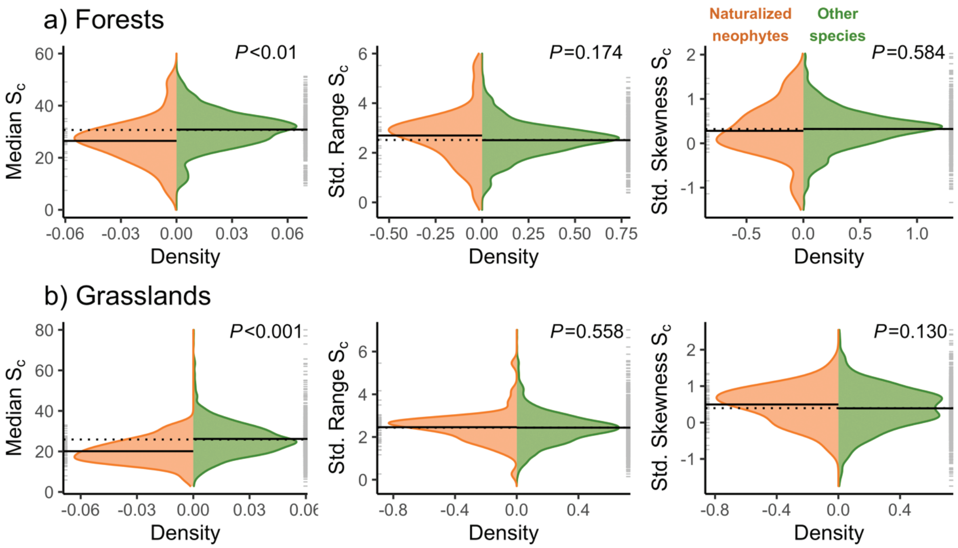
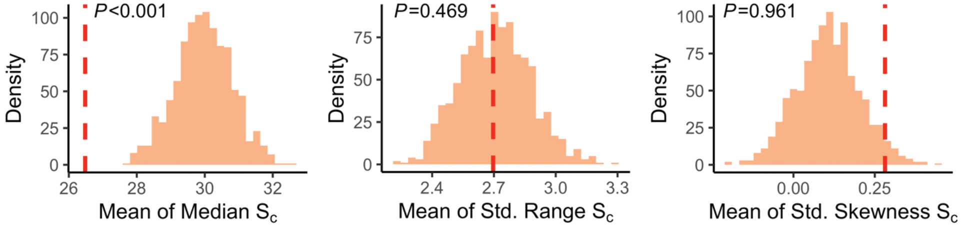


Figure 2. Density curves comparing the median (1st column), range (2nd column) and skewness (3rd column) of plot-size adjusted species richness (S_c) of naturalised neophytes with all other species in (**a**) forests and (**b**) grasslands. The dotted black line indicates the mean of S_c values for each parameter across all species in the vegetation formation. The solid black line indicates the mean of the S_c values for each parameter of each species group. The tick marks on the left and right margins show the S_c values for each parameter of individual species in each group. Density values of naturalised neophytes were multiplied by -1 to facilitate visual comparisons. The range and skewness of S_c were standardised (Std.) as described in the Methods section. *P*-values correspond to Mann-Whitney U Tests.

a) Forests



b) Grasslands

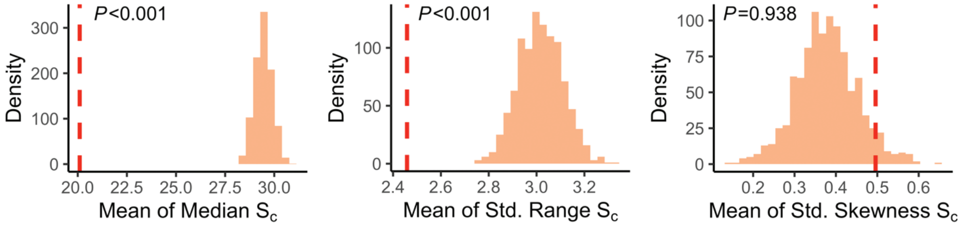


Figure 3. Comparison of mean observed values of the median (1st column), range (2nd column) and skewness (3rd column) of S_c of naturalized neophytes with the distribution of mean random values of the same parameters obtained from the null model. Results are for species in (**a**) forests and (**b**) grasslands. The dashed red line represents the mean observed value of each parameter across all species. The bars show the distribution of random values of each parameter. The range and skewness of S_c were standardised (Std.) as described in the Methods section. *P*-values indicate the proportion of the randomised parameters that are lower than the observed value.

Comparing S_c of naturalized neophytes against the random expectation (RQ2)

Naturalised neophytes tended to occur more frequently in communities with fewer species than expected by chance, both in forests (Fig. 3a) and grasslands (Fig. 3b). Naturalised neophytes also tended to occur in communities with a narrower range of richness values than under random expectation in grasslands. The symmetry in the distribution (skewness) of richness values of naturalised neophytes did not differ significantly from the random expectation.

Changes in S_c of naturalised neophytes amongst clades (RQ3)

Naturalised neophytes generally had lower than expected median S_c values (64%), particularly in grasslands (83%) (Fig. 4). Only *Reynoutria japonica* and *Aesculus hippocastanum* occurred in forest communities with more species than expected by chance. Fabaceae species generally did not deviate from the random expectation in terms of median S_c. Some clades also showed contrasting patterns between forests and grasslands. For example, the median S_c of naturalised neophytes from the Asteraceae family did not deviate from random in forests, but they showed lower median S_c than under random expectation in grass-

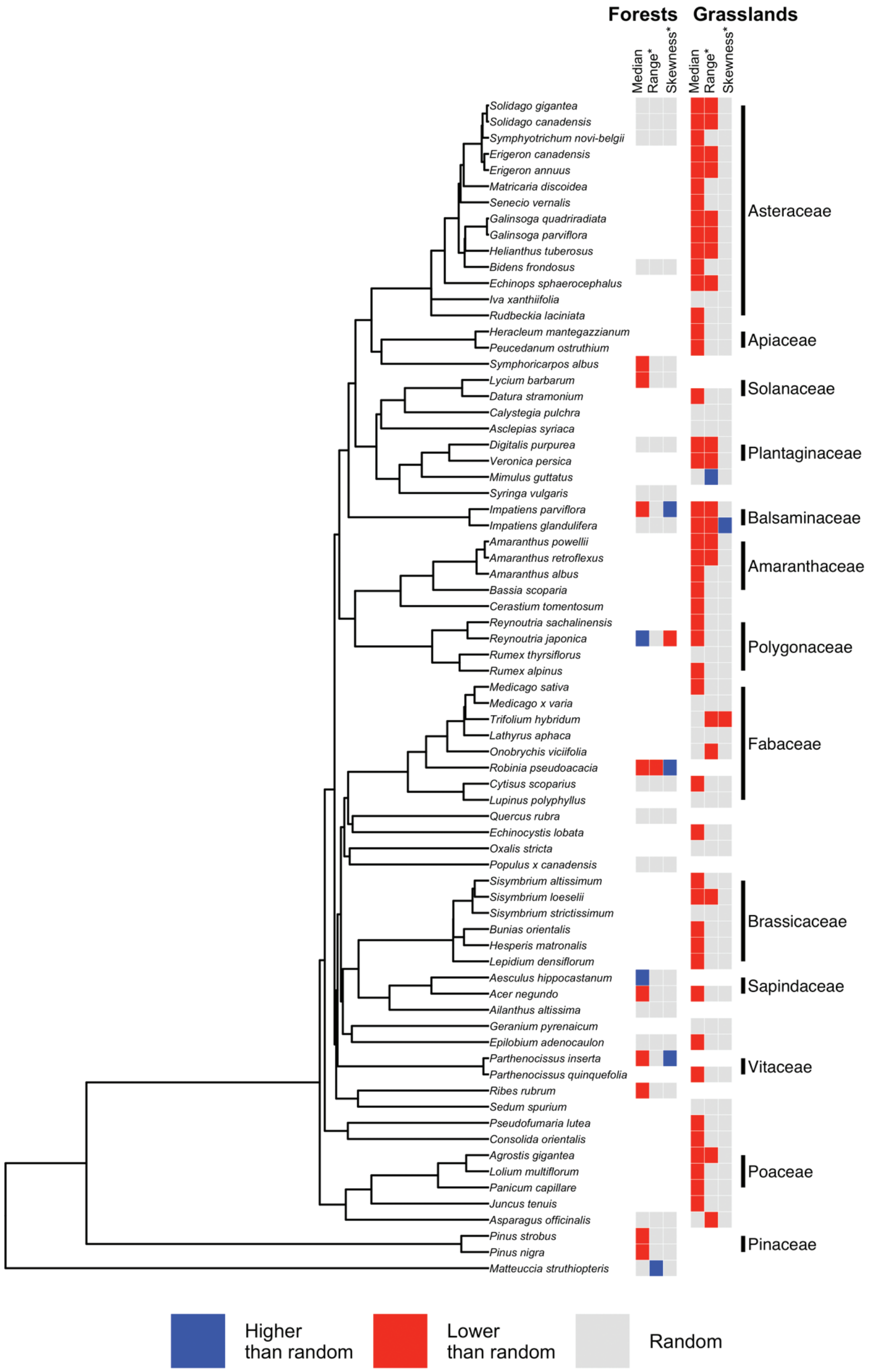


Figure 4. Phylogeny of naturalised neophytes found in vegetation plots. For each species, we show whether the median, range and skewness of S_c were higher (blue) or lower (red) than under random expectation or did not differ from the random expectation (grey) in forests and grasslands. The range and skewness of S_c were standardised as described in the Methods section. * = Standardised.

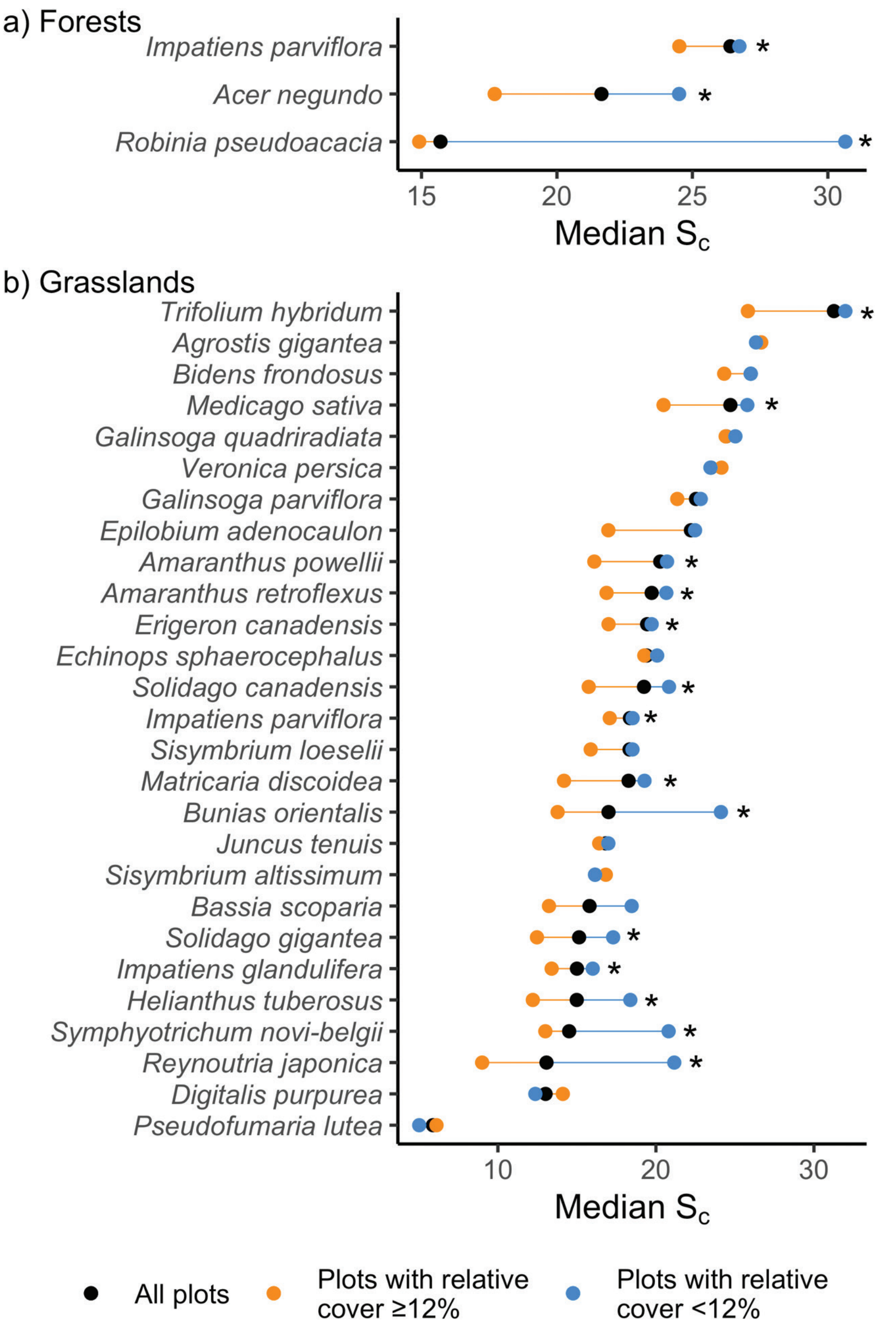


Figure 5. Median S_c of naturalised neophytes across all plots where they occurred and in plots with relative cover greater or smaller than 12%. Species are classified, based on their occurrence in (**a**) forests and (**b**) grasslands. Only species that occurred in at least ten plots in each group of plots (i.e. those with relative cover ≥ 12% vs. those with relative cover < 12%) were considered. Asterisks (*) indicate significant ($P < 0.05$) differences in median S_c between groups following Mann-Whitney U Tests. Median S_c values and number of plots associated with each species can be found in Suppl. material 1: Appendix S5: Table S5.2.

lands (see also *Digitalis purpurea*, *Impatiens glandulifera* or *Epilobium ciliatum*). About 31% of species in communities with a lower number of species than randomly expected also had a lower range of species richness than randomly expected. Only 7% of species that occurred in communities with a lower number of species than randomly expected were also more positively skewed towards species-poor communities than randomly expected.

Variation in S_c of naturalised neophytes with contrasting relative abundance (RQ4)

We found that naturalised neophytes generally had higher abundance (relative cover ≥ 12%) in communities with lower numbers of species than in communities with higher numbers of species in both forests and grasslands (Fig. 5). The most remarkable differences in median S_c of plots dominated (relative cover ≥ 12%) and not dominated (relative cover < 12%) by individual naturalised neophytes were for *Robinia pseudoacacia*, *Acer negundo* and *Reynoutria japonica*. Naturalized neophytes that frequently had low relative cover within communities (i.e. median S_c of all plots was similar to median S_c of plots with relative cover < 12%) included, for example, *Impatiens parviflora*, *Amaranthus retroflexus*, *Erigeron canadensis*, *Sisymbrium loeselii* and *Matricaria discoidea*. In contrast, naturalized neophytes that frequently had high relative cover within communities (i.e. median S_c of all plots was similar to median S_c of plots with relative cover ≥ 12%) included *Robinia pseudoacacia*, *Bunias orientalis* or *Symphyotrichum novi-belgii*. Although fewer naturalised neophytes reached a relative cover ≥ 25% in plots, we found similar results when we increased the cut-off for dominance to this value (Suppl. material 1: Appendix S5).

Discussion

Using a dataset spanning over a broad geographic area, we have demonstrated that the relationship between the occurrence of alien species and the diversity of local plant communities is species-dependent and varies by habitat. However, when considered together, alien species occur more frequently in species-poor communities than expected by chance. Alien species also occur in species-poorer communities than the rest of the flora in the Czech Republic. These patterns are more pronounced in grasslands, where alien species also occur in communities with a shorter diversity gradient (narrower range of richness) than would be expected by chance.

We suggest that the negative association between the occurrence of most alien species and community diversity in our study may be due to two main mechanisms. First, according to the biotic resistance hypothesis, diverse native communities might resist invasion by competition, herbivory and pathogens (Elton 1958; Lonsdale 1999; Jeschke 2014). Conversely, species-poor communities in stressed environments might have more empty niches available for alien species (MacArthur 1970). Second, alien species might initially establish in species-rich communities, but then cause local extinctions of resident native species, leading to a decline in overall diversity (Hejda et al. 2009).

It is likely that these two mechanisms act simultaneously to influence the association between alien species and community diversity. *Matricaria discoidea*, *Erigeron canadensis* and *Amaranthus retroflexus*, the three species associated with the lowest standardised median species richness in grasslands, grow primarily in species-poor ruderal vegetation, where they take advantage of gaps caused by various disturbance events to establish (Pyšek et al. 2009). Similarly, *Impatiens parviflora*, which was also associated with one of the lowest standardised median species richness in our study, invades most efficiently in disturbed forests with depauperated herb-layer communities, whereas more natural forests with species-rich herb layers appear to be more resistant to invasion by this neophyte (Obidziński and Symonides 2000). Previous studies have shown that invasive *Impatiens* species in the study area (*I. parviflora* and *I. glandulifera*) have only a minor impact on the native species diversity of invaded forests' herb layers (Hejda et al. 2009; Hejda 2012).

In contrast, some dominant invasive alien trees, such as *Robinia pseudoacacia* or *Pinus strobus*, inhibit understorey vegetation growth and native tree regeneration through a combination of effective seed dispersal, high seedling recruitment, fast growth or alteration of soil conditions (Hadincová et al. 2007; Cierjacks et al. 2013; Vítková et al. 2017). *Reynoutria japonica* is associated with species-poor communities in grasslands, but species-rich communities in forests. This difference can be partially explained by the fact that *R. japonica* more frequently invades riparian habitats, which are amongst the most species-rich forests in the study area, despite the potentially adverse effect of this species on community diversity (Hejda et al. 2021). However, *R. japonica* often becomes a strong dominant in herbaceous vegetation, overgrowing and out-competing other species (Hejda et al. 2009). Most herbaceous alien species from the Fabaceae family showed random associations with species diversity in grasslands. It is possible that these alien species do not occur in species-poor communities because they escape competition using nitrogen fixation through bacterial symbiosis and indirectly enrich the soil, allowing more species to become established (Sprent 2007). Nonetheless, most herbaceous legumes have also been actively sown, increasing the probability of their establishment regardless of the competition in the community. In general, alien species were associated with less species-rich communities when they exhibited high abundance, a pattern commonly observed in studies of native-invasive species interactions (Bradley et al. 2019).

We characterised individual naturalised alien species by calculating three key parameters (median, range and skewness) of the distribution of species richness of the communities in which the species occurred and compared these values to the null expectation to test if alien species assembled at random. As in previous studies, we confirmed that naturalised alien species generally occur in relatively species-poor communities (e.g. Fridley et al. 2007; Peng et al. 2019), but we also extend previous work by confirming that they can colonise species-poor communities with a narrower range in species number than expected by chance. This pattern was more evident in grasslands than in forests, indicating higher habitat specialisation of alien species in certain grassland types (Chytrý et al. 2008; Axmanová et al. 2021), perhaps reflecting the fact that our grasslands encompassed a broader range of vegetation types than forests. However, our species-level analyses showed that the associations of alien species with species-poor communities are not always accompanied by associations with a more restricted range of species richness or greater asymmetry in their distribution. This coupling only occurred in two species (*Impatiens glandulifera* and *Robinia pseudoacacia*), which can be considered two highly specialised species that thrive under disturbance and eutrophic conditions in particularly species-poor sites. Further studies should also consider changes in beta-diversity at different levels of invasion to better characterise the relationships between alien species and community diversity and infer the impact of biological invasions on community structure. Such an approach will make it possible to examine whether individual alien species, belonging to particular clades, reduce community diversity and homogenise their composition when they become dominant.

To date, most studies examining native-alien species interactions had been conducted either at the plot level (e.g. Stohlgren et al. 2003, 2006; Boughton et al. 2011) or for a subset of alien species (e.g. Hadincová et al. 2007; Hejda et al. 2009, 2021), usually in a relatively small geographical area (Peng et al. 2019). A major strength of this study is that we calculated scores of individual species for their tendency to occur in species-poor or species-rich communities over a large area. Our analyses included invaded and non-invaded plots representing natural vegetation in the Czech Republic. Both invaded and non-invaded plots were combined in a null model approach to quantify the frequency, strength and consistency of non-random species associations. Although this approach assumes that all species in the regional species pool of each vegetation formation (i.e. forests and grasslands) can occur in plots of the same vegetation formation throughout the country, it allowed us to detect the signature of non-random mechanisms of community assembly and invasion. Our approach offers additional advantages over the methods used in the above studies when analysing large co-occurrence datasets and it could also be applied to other types of systems. For example, our approach could be used to test whether endemic or endangered species occur in species-rich or species-poor communities and ultimately provide information for conservation policy.

This study is one of the most comprehensive quantitative analyses to date examining the relationship between alien plants and the species richness of resident vegetation. The 73 alien species included in the study are considered invasive in most Central-European countries (Axmanová et al. 2021) and can be representative of plant invasions in the temperate zone. Europe has been a centre for international trade for many centuries, introducing many alien species to the continent (van Kleunen et al. 2018). This research also shows that multi-species studies are needed to understand the processes of community assembly and to assess the impact of alien plant invasions on native diversity. Supplementing our conclusions with results from long-term experimental community studies could provide further insights into the role of underlying factors driving biological invasions.

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Supplementary material 1

Alien plants tend to occur in species-poor communities

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Data type: Docx file.

Explanation note: **Appendix S1**: Overview of vegetation plots included in the different vegetation types. **Appendix S2**: Correlations between the median, range, and skewness of S_c. **Appendix S3**: Results for invasive neophytes. **Appendix S4**: The S_c statistics of individual naturalized neophytes. **Appendix S5**: Results considering a cut-off of 25% of cover to determine dominance.

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Early-phase colonisation by introduced sculptured resin bee (Hymenoptera, Megachilidae, *Megachile sculpturalis*) revealed by local floral resource variability

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Abstract

There is a growing interest to document and better understand patterns and processes involved in non-native bee introductions and subsequent colonisation of new areas worldwide. We studied the spread of the East Asian bee *Megachile sculpturalis* in Serbia and south-eastern Europe; the bee was earlier established in the USA (since 1994) and western Europe (since 2008). Its establishment in Serbia remained dubious throughout most of 2017–2019, following its first detection. We hereby report on its establishment and spreading, which were corroborated in 2019 under specific circumstances. Owing to an exceptionally poor blooming of *Styphnolobium japonicum* in 2019, we recorded a high activity density of *M. sculpturalis* concentrated on a scarce key food resource. We present a novel quantitative approach for an improved early detection of *M. sculpturalis*, based on the interplay between the bee local occurrence pattern and dynamics of key food-plant(s) availability. This approach seems particularly effective during the early-phase colonisation, at initially low population density of introduced bees. We address the importance of integration of the genuine plant usage patterns with context-specific bee assessment options in establishing effective monitoring. The improved understanding of *M. sculpturalis* local dynamics triggered the questions about possible origin(s) and modes of its dispersal east of the Alps. To explore the possible scenarios of *M. sculpturalis* introduction(s), we extended the study to a wider spatio-temporal context – the region of SE Europe (2015–2019). The two complementary study approaches (at local and regional scale) provided more comprehensive evidence of bee dispersal history and the detection patterns in varied recording contexts. Based on this two-scale approach, we suggest that a diffusive mode of *M. sculpturalis* introduction into Serbia now seems to be a more plausible scenario (than a long-distance jump). We argue that the integration of outcomes from the contrasting approaches (a systematic surveillance, based on plant resources and a broad-scale opportunistic recording) could be of great methodological relevance for the development of future monitoring protocols.

Keywords
colonisation scenarios, invasive pollinators, monitoring, non-native bees, Serbia, south-eastern Europe, *Styphnolobium japonicum*

Introduction

Amongst the continually growing number of introduced species being discovered around the world (Seebens et al. 2017; Pyšek et al. 2020), bees as a group (Hymenoptera, Apoidea, Anthophila) may count as moderately large. There are about 80 species worldwide found outside their native ranges (Russo 2016), representing a small share of an estimated total of more than 20,000 extant bee species (Michener 2007; Ascher and Pickering 2020). The majority of non-native bees (around 73%) were established following accidental introductions, with a remarkable number of those that are cavity-nesters, principally in various types of wooden material or hollow plant stems (Russo 2016; Poulsen and Rasmussen 2020). Most of the introductions happened in North America (around 69%), while Europe could be currently regarded as a comparatively "coldspot" of alien bee species richness. Of only three fully-confirmed non-native bee species in Europe, two are still known from quite restricted areas: *Megachile disjunctiformis* Cockerell, 1911, in central Italy (only Bologna) and *Xylocopa pubescens* Spinola, 1838, in southern Greece and southern Spain (Rasmont et al. 2017; Bortolotti et al. 2018). So far, *Megachile sculpturalis* Smith, 1853 is the only alien bee widely established and continuously spreading across Europe.

A growing number and geographical extent of alien bee introductions worldwide raise concerns regarding their potential to cause negative environmental impacts. Documented or predicted impacts include: decline of native bee populations through competition (for floral or nesting resources) or pathogen and parasite transmission, degradation of native flower-pollinator networks, reduced pollination of native and crop plants, facilitation of alien weeds and invasive plants (Goulson 2003; Russo 2016; Morales et al. 2017; Vanbergen et al. 2018; Aizen et al. 2020). So far, detrimental effects caused by alien bees were clearly established in a relatively few cases – principally by representatives of social bees: *Apis mellifera* L. and *Bombus* spp. (Russo 2016; Morales et al. 2017). Much less common are studies that quantitatively document possible negative impact of solitary alien bees (MacIvor and Packer 2015; Fitch et al. 2019; LeCroy et al. 2020), including the recent survey on *M. sculpturalis* (Geslin et al. 2020). Although these results were mostly based on correlational evidence, the lack of indisputable interaction amongst native and non-native bees should not be interpreted as the lack of impact (Stout and Morales 2009). In the case of *M. sculpturalis*, its most worrying feature is an unusually aggressive and/or destructive behaviour exerted while competing with native solitary bees for nesting cavities (summarised in: Le Féon et al. 2018; Lanner et al. 2020a, 2020b; Straffon-Díaz et al. 2021). This competition may be both direct (aggressive repelling or even destruction of brood of other bees in already closed nests) or indirect (exploitative competition for nesting cavities), including the elements of amensalism (e.g. killing of co-occurring species that are using the cavities of different size-classes, i.e. not being in competition). However, there are still no exact and straightforward estimates of its extended impact on affected taxa, i.e. the conclusive evidence of causative effects on population trends. Following the environmentalist's precautionary principle, it may be best considered as a potentially invasive alien species (Bila Dubaić et al. 2021), pending further studies. In this context, we use the term "invasive" in its restricted conservationist sense, i.e. to denote a subset of all established alien species that cause substantial negative impact on native biota and/or ecosystems (e.g. Russo 2016; Vanbergen et al. 2018; IUCN 2020). In some other studies on this bee, the term was used somewhat more loosely or in a wider sense – according to the approach to include all alien species that are rapidly/extensively expanding in the non-native range, regardless of impacts (cf. Blackburn et al. 2011; Hui and Richardson 2017; for conceptual and terminological controversies in invasion biology, see also: Hoffmann and Courchamp 2016; Courchamp et al. 2017).

Megachile sculpturalis belongs to the subgenus *Callomegachile* Michener, which is distributed principally in the Old-World tropics (Michener 2007; Ascher and Pickering 2021). As a rare extratropical member, *M. sculpturalis* range stretches across eastern Asia: from Taiwan and eastern mainland China, through Korean Peninsula to Japan (Batra 1998; Wu 2006; Ascher and Pickering 2021). It is a bee of a distinctive appearance – quite large, parallel-sided black body, 21–27 mm long in females and 12–22 mm in males (Hinojosa-Diaz et al. 2005; Aguado et al. 2018), wings transparent with dark tips (a comprehensive description is available in Sheffield et al. 2011). Females make nests in existing cavities in dead wood or hollow plant stems and seal them with resin-like material (Hinojosa-Diaz et al. 2005; Maeta et al. 2008; Quaranta et al. 2014; Westrich et al. 2015), hence the colloquial name initially coined for this bee in North America: "giant resin bee"; here, we adopted a more appropriate alternative name: "sculptured resin bee" (from: iNaturalist.org). It is assumed that long-distance overseas introductions of this cavity nester have happened via inadvertent importation of timber goods or other suitable nesting materials (Mangum and Brooks 1997; Quaranta et al. 2014; Russo 2016; Le Féon et al. 2018). A similar mechanism (passive, human-assisted transportation) is expected to contribute to secondary introductions within colonised continents, as long- or short-distance jumps (Westrich et al. 2015; Lanner et al. 2020a). Due to a large size, contrasting dark and bright colour-pattern, vivid nesting activity and frequent visitation of some commonly-available ornamental plants, this bee is readily spotted and recognised and, hence, expectedly easy to be detected as soon as it appears in new areas (Quaranta et al. 2014; Lanner et al. 2020a).

The first confirmed establishment outside of *M. sculpturalis* native range was in 1994 in North Carolina, USA (Mangum and Brooks 1997), followed by a rapid expansion across the eastern half of North America (Mangum and Sumner 2003; Hinojosa-Diaz et al. 2005; Parys et al. 2015). In Europe, it was first detected in 2008, in south-eastern France (Vereecken and Barbier 2009); this was soon followed by records from north-western Italy in 2009 and south-eastern Switzerland in 2010 (Amiet 2012; Quaranta et al. 2014). Documented spreading in the period 2011–2014 was mostly confined to these areas (Westrich et al. 2015; Le Féon et al. 2018; Ruzzier et al. 2020). Since 2015, a more dynamic range extension took place: around the Alps in Switzerland, southern Germany, Slovenia, Austria and Liechtenstein (Westrich et al. 2015; Dillier 2016; Gogala and Zadravec 2018; Lanner et al. 2020a; Westrich 2020), much more widely in France and Italy (Le Féon and Geslin 2018; Le Féon et al. 2018; Poggi et al. 2020; Ruzzier et al. 2020), south-westwards into NE Spain (Aguado et al. 2018; Ortiz-Sánchez et al. 2018) and, most recently, across the Mediterranean Sea, reaching the Island of Mallorca as the southernmost point in Europe so far (Ribas Marquès and Díaz Calafat 2021). In contrast with this largely continuous spreading (comprising also few smaller jumps), the range establishment east of the Alps (2015–2019) represents more remote, arguably long-distance dispersal events of yet unclear origin and mechanisms: into north-eastern Hungary (Kovács 2015), north-eastern Austria (Westrich 2017), northern Serbia (Ćetković and Plećaš 2017), southern Croatia (Resl 2018; "pitrusque" 2019), Crimea (Ivanov and Fateryga 2019), southern Romania (Hymenopterists Forum 2019) and northern Bosnia & Herzegovina in 2020 (Nikolić 2020). The range expansion within Europe for the reference period (2008–2019) is presented schematically with a series of phase-maps and a summarising review of its dynamic distribution (Ćetković et al. 2020; Suppl. material 1).

Following its remarkable non-native spreading, evidence was accumulated about sculptured resin bee interactions with numerous plant genera and families (Quaranta et al. 2014; Parys et al. 2015; Le Féon et al. 2018; Ruzzier et al. 2020), showing a more diverse spectrum than currently available for its native area. Accordingly, *M. sculpturalis* is often referred to, or assumed to be polylectic (Mangum and Brooks 1997; Maeta et al. 2008; Quaranta et al. 2014; Parys et al. 2015; Westrich et al. 2015; IUCN 2020; Ribas Marquès and Díaz Calafat 2021), but without clear evaluation of the documented visitation pattern, for example, the share of pollen vs. only nectar foraging. Based on numerous sources (cf. Ćetković et al. 2020; an ongoing analysis), *M. sculpturalis* shows a particularly strong preference for the pollen of Fabaceae (Batra 1998; Mangum and Sumner 2003; Maeta et al. 2008; Westrich et al. 2015; Campbell et al. 2016; Dillier 2016; Hall and Avila 2016; Aguado et al. 2018; Andrieu-Ponel et al. 2018; Le Féon and Geslin 2018; Le Féon et al. 2018; Guariento et al. 2019; Ruzzier et al. 2020; Westrich 2020; Ribas Marquès and Díaz Calafat 2021), principally of large-flowered members of the subfamily Faboideae. Within the European range, the most frequently documented pollen-source is the ornamental Japanese pagoda tree, *Styphnolobium japonicum* (L.) Schott. In contrast with this growing evidence, the loosely repeated "wide polylecty" might represent a biased or uncritical perception, which ignores often remarkably narrow effective dietary preferences of *M. sculpturalis*. The patterns of genuine plant usage of this bee are yet to be thoroughly evaluated, taking into account the floral resource availability, the variability of plant phenology, as well as the suitability of different habitat types across the colonised areas. Undoubtedly, *M. sculpturalis* is quite opportunistic in its foraging for nectar sources, but much more selective when provisioning the pollen for its brood.

Despite a growing number of studies, a specified approach is still missing to quantify the sculptured resin bee distribution dynamics and population trends, its interactions with key plants and native bees and, hence, ultimately, to assess its invasive potential. As a first step, we need an effective approach for early detection and extended surveillance of its expansion. To address these questions, we explored the spatial relationships between bee activity patterns and local availability of key plant resources. We present the survey of the sculptured resin bee introduction in Serbia as an event-driven case study of an early-phase colonisation. Initially, the accidental encounter of a single specimen early in 2017 was interpreted as a likely long-distance chance dispersal of uncertain success (Ćetković and Plećaš 2017). After an extended lack of confirmation throughout the seasons of 2017–2018, the current study was initiated with the second find in the same area (the city of Belgrade), attained late in 2019 under specific circumstances: owing to an extremely reduced blooming of its preferred host plant (*S. japonicum*) in that year, we managed to detect a vivid activity of still locally-rare sculptured resin bees, temporarily concentrated on a scarce food source. We evaluated the effects of variable floral resources on the sculptured resin bee detectability and developed a quantitative approach for an integrated assessment of the plant availability and bee local density, as a step towards a more comprehensive monitoring. The improved understanding of *M. sculpturalis* local dynamics further triggered the questions about the origin(s) and modes of its dispersal east of the Alps (since 2015; cf. above), which were not appropriately addressed elsewhere. Therefore, we extended the study to a wider, regional-scale context, to consider possible scenarios of *M. sculpturalis* introduction(s) and to reveal its early colonisation dynamics within the SE Europe region. The two-scale study (local and regional) should provide a much wider complementary evidence from the methodologically different approaches across the spatio-temporal scales, aiming to relate the bee dispersal history to the detection patterns on a more comprehensive basis. Therefore, we expect that outcomes would be highly relevant for the development of standardised monitoring protocols.

Material and methods

The study of *M. sculpturalis* arrival and establishment in Serbia was mostly based on extensive fieldwork within the city of Belgrade, during the period of 2017–2019. The wider geographical and temporal context of this survey included principally the eastern Pannonian Plain, but we also considered the nearest known occurrences towards the west and east of this area (from Austria and Slovenia through the Crimean Peninsula), for the period of 2015–2019.

Local scale: Belgrade as the study area

Belgrade is one of the largest cities in south-eastern Europe (Belgrade "proper" administrative-urbanistic core area is nearly 776 km^2, population > 1.5 million), situated at the border between the two quite different geographical units: the predominantly hilly to mountainous Balkan Peninsula to the south and the vast lowlands of the Pannonian Plain to the north. It is positioned in a climatically transitional zone between the temperate-continental and the more steppic regime, with a relief spanning the altitude range of 65–506 m. The Belgrade area encompasses more than 50% of varied agricultural habitats as a matrix, with embedded mosaics of urban and rural habitats; two principal sections of Belgrade (the Balkan and the Pannonian – Fig. 1) are characterised by a distinct spatial arrangement and contrasting types of settlements, agricultural systems and more natural habitats, owing to a largely different physiography and historical development. From the perspective of wild bee studies, various urbanistic areas may be characterised by differing types, extent and relative share of suitable habitats (e.g. from urban green to semi-natural). In order to consider possible coarse-scale effects of variability in key resources and other environmental features across urban gradients of Belgrade, we used a framework of wider "urbanistic zones" (Fig. 1). They are based on landscape scale characterisation of available elements of physiography, land-cover, gradients of urbanisation and management regime features. Some easily-defined coarse-scale differences (e.g.

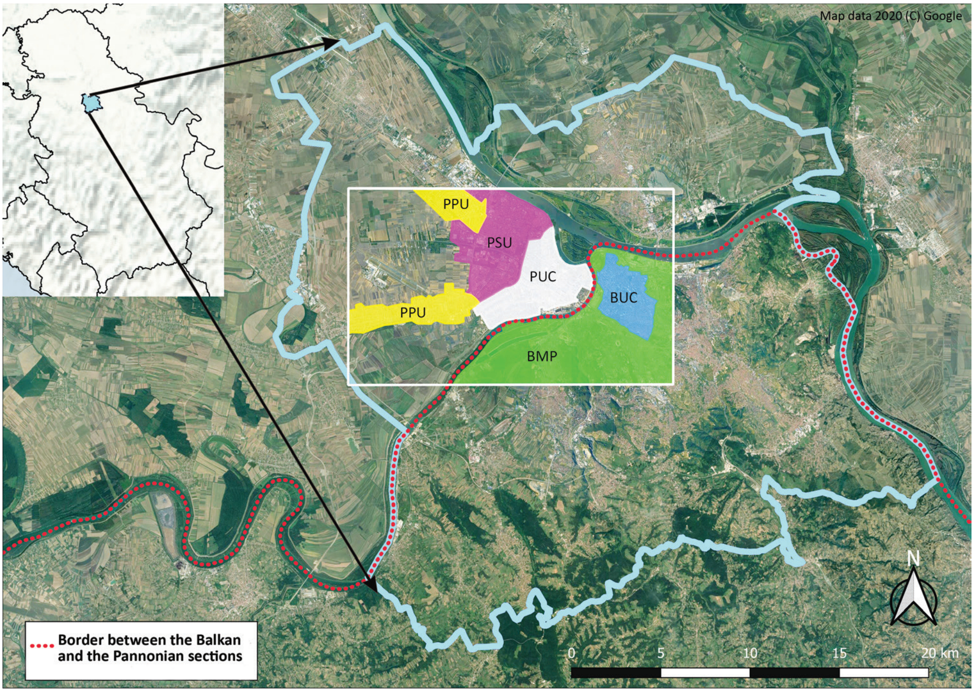

Figure 1. Landscape/urbanistic zonation of the study area in Serbia (18×11 km), within Belgrade proper (light blue outline; sections separated by the red dotted-line): BUC – Balkan Urban Core; BMP – Balkan Mixed Periphery; PUC – Pannonian Urban Core; PSU – Pannonian Semi-Urban; PPU – Pannonian Peri-Urban.

varied urban temperature regimes or dominant management practices) might differently affect activity patterns of plants and bees, potentially leading to dynamic shifts in bee local distribution and resource usage. More details on the wider study area and operative aspects of zonation used in this survey are available in Suppl. material 2: Figs S2.2–S2.23.

Local scale: survey set up and study design

The first record of *M. sculpturalis*, in early July 2017 (a single male), was an unexpected find within a routine monitoring of wild bee communities of selected urban habitats in the Belgrade area (Ćetković and Plećaš 2017; Fig. 2A). However, its establishment in Serbia remained unconfirmed during the first two seasons. We extended efforts to explore the state of its presence, focusing on locations with the Japanese pagoda tree (*Styphnolobium japonicum*), which is the most favourable pollen source for *M. sculpturalis* in Europe. This exotic plant is probably the only species with both an appropriate blooming phenology and a high density throughout the Belgrade area. During the seasons of 2017–2018, our surveys covered 12 locations within 18 days (26 "occasions" = unique date/location combinations), spending about 440 person-minutes (= 7.25 person-hours) in collecting or observing bees on *S. japonicum* throughout the area and covering the *M. sculpturalis* main flight period. At the same time, we continued with variously focused wild bee surveys on other abundant summer-blooming plants across Belgrade. This included an extensive survey on *Lavandula* and *Ballota* (Lamiaceae) and sporadically on *Buddleja* (Scrophulariaceae) – all being listed as attractive food plants for *M. sculpturalis*, at least as nectar sources (Quaranta et al. 2014; Le Féon et al. 2018; only *Ballota* was documented as a pollen source, cf. Ivanov and Fateryga 2019). For observations on *Lavandula* (8 locations within 18 days, 21 unique occasions, totalling nearly 490 person-minutes) and *Ballota* (13 locations within 27 days, 32 unique occasions, totalling > 1,190 person-minutes), we spent about 28 person-hours during the summer seasons of 2017–2019. None of these efforts yielded any additional point-occurrences or recorded interactions of *M. sculpturalis* (for *Buddleja* see Results).

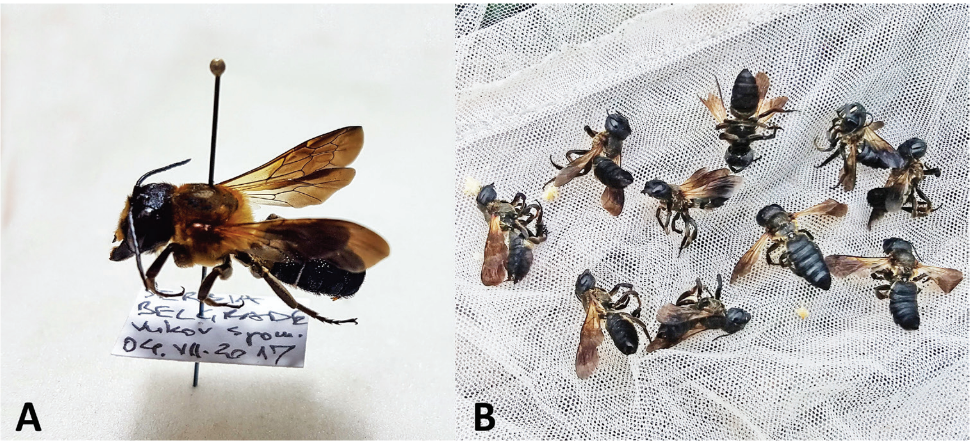

Figure 2. A the first specimen of *Megachile sculpturalis* (male), caught in Serbia in July 2017 **B** mass-foraging females detected in August 2019.

The second record of *M. sculpturalis* was also accidental. The summer of 2019 was characterised by an extreme failure in *S. japonicum* blooming (see details in Results); for this reason, this plant was excluded from our regular monitoring that year. Then, upon an unexpected detection of numerous sculptured resin bees on 2 August 2019, on a single *S. japonicum* tree (Fig. 2B; Suppl. material 3: Table S3.2), we undertook an immediate survey across Belgrade to document and quantify its confirmed presence. We searched for and checked as many locations with *S. japonicum* as possible, across all urbanistic zones (Fig. 1; see also: Suppl. material 2: Fig. S2.3). However, the next 7-day period (3–9 August) represented the very end of the *S. japonicum* blooming season in 2019, when many trees had already ceased blooming.

On all locations with still-blooming trees, we conducted estimation of bees foraging on flowers, using binoculars where needed (for high crowns). Due to different situations amongst the sites as well as logistic constraints, the duration of work at each location varied from 1–50 minutes (mostly ranging 10–20, mean ~ 15.3 ± 10.7 SD). The estimation procedure was adjusted to varied levels of activity density: (**a**) at sites with lower activity (≤ 5 observable individuals), bees were usually not present continuously during the observation; here, we used timed counts to quantify the presence of foraging bees on a tree and, if the number of individuals was changing over the period of observation, we split the total time into intervals characterised by each recorded value (0–5); (**b**) when continuous and more vivid activity was observed (> 5 bees visible at any moment), 3–4 snapshot counts were made over the time spent on site, using two abundance classes: moderate (6–10) or high (11–20). We adapted the snapshot technique used in ornithology (Gaston et al. 1987; Greene and Efford 2012; Barraclough 2020), which proved suitable for situations when numerous individuals are flying within the field of view, without the possibility to count them accurately. At a few sites with variable bee activity on different trees, the combination of both techniques was employed. To enable standardised comparisons, we scaled all recorded values to one minute of continual bee activity on a defined unit of floral resource within a landscape sector (as elaborated further on), by averaging all counts against the recorded time (hereafter Bees per Minute = BpM). Details of recording and calculation procedures are available in Suppl. material 3 (explanation of metrics in Table S3.1; sampling duration and BpM estimates per sectors in Table S3.2). In total, we spent about 300 person-minutes (5 person-hours) working on 16 sites with at least some blooming trees (out of 40 surveyed sites), mostly observing and counting (> 260 minutes). At some sites, we also collected bee specimens by hand-net, as vouchers and for future genetic studies.

Simultaneously, we estimated the key floral resource to assess if its quantity, distribution and phenology affect the local differences in activity density and distribution of the bee population. We recorded the number of *S. japonicum* trees (hereafter NoT) and visually assessed their actual blooming status at each visited location: the number of trees that entered blooming in 2019 (hereafter NoT_iB), the share of inflorescences developed on each crown in bloom during 2019 (as a fraction of the fully-blooming crown; summed value interpreted as Total Floral Resource, hereafter TFR) and, finally, the actual share of flowers still in bloom on each crown at the moment when we made the observation (summed to Current Floral Resource, hereafter CFR). We continued to survey *S. japonicum* until early September, regardless of the ceased blooming (and no bee activity), to provide the spatial coverage of resource availability across the study area. For extended explanations and visual examples of these parameters, see in Suppl. material 3: Table S3.1.

All surveyed locations were primarily georeferenced in Google Earth Pro ver. 7.3.3.7786 (Google Inc. 2020) and further prepared as distribution maps in QGIS ver. 3.4 (QGIS Development Team 2018). To deal with the uneven and patchy distribution of surveyed *S. japonicum* trees and the logistic limitations of the sampling approach, we grouped the point-sampled quantitative data following the rationale similar to landscape ecology studies on wild bees (e.g. Steffan-Dewenter et al. 2002; Steckel et al. 2014; Cohen et al. 2020). We defined a primary framework of circular sectors with 250 m radius (hereafter: S250; Suppl. material 2: Fig. S2.3A), manually fitted to include all surveyed point-locations without overlapping. Various bees perceive the landscape composition and configuration (particularly distribution of resources and other habitat features) at different spatial scales, since their foraging ranges depend principally on size; the radius of 250 m is commonly used to define the smallest meaningful study scale (Steffan-Dewenter et al. 2002), while larger-bodied bees may forage at much larger distances (Gathmann and Tscharntke 2002; Greenleaf et al. 2007). Due to spatial limitations of the sampled area, we added only one coarser scale (sectors of 500 m radius, hereafter S500; Suppl. material 2: Fig. S2.3B) to test for different scale effects. As a result, all recording sites were arranged into two series of standardised circular sectors: 40 locations S250 (ca. 0.2 km^2) and 23 locations S500 (ca. 0.8 km^2); all parameters were calculated per those spatial units (see more details about the procedure in: Suppl. material 2: Fig. S2.3). Therefore, we used some technical concepts and experiences from landscape ecology studies as a suitable practical approach (and a prospective “working framework”) to quantify and analyse relationships between bee distribution patterns and resource availability.

All values from field assessments were summed per defined sector. To calculate TFR, we summed individual values from each *S. japonicum* tree in bloom, expressed as a fraction of the whole crown, based on the estimated maximal extent of blooming attained during the summer of 2019. Similarly, we calculated CFR as a sum of estimated blooming fractions at the moment of assessment; this represents the actual extent of blooming of each crown within the sector. We recorded blooming fractions as percentage of the whole crown for each assessed tree and then summed the values in decimal form (e.g. blooming of 10% of one crown, 25% of another and 80% of a third gives the value of 1.15 “unit crowns” per sector; more details and visual examples for the calculation available in Suppl. material 3: Table S3.1, summed results in Table S3.2).

Local scale: statistical analyses

We tested if various aspects of floral resource distribution and seasonal dynamics (i.e. change from TFR to CFR level of blooming) had a measurable effect on local differences in bee activity. We analysed the relationship between the bee activity density (BpM) and all measured parameters of the key floral resource (NoT, NoT_iB, TFR and CFR), calculated in S250 and S500 frameworks, with the Generalised Least Square (GLS) linear regression, to account for heteroscedasticity of errors. Additionally, we used GLS linear regression to analyse the relationship between BpM and TFR, CFR, percentage of TFR (TFR/NoT) and percentage of CFR (CFR/NoT), all averaged for each urbanistic zone. Analyses assumptions were tested by examination of residuals. Furthermore, we tried to establish if there were any local patterns in reduction of *S. japonicum* blooming (i.e. any possible differences caused by environmental effects that specifically vary with urbanistic gradients, using urbanistic zones as tentative proxies) and, if so, are the bees responding to these differences. Differences in NoT, NoT_iB, TFR, CFR and BpM between urbanistic zones were analysed by the Kruskal-Wallis test. All analyses were performed in R v.3.6.3 (R Core Team 2020) and the R-package nlme v.3.1-144 (Pinheiro et al. 2020).

Regional context: introduction and expansion within SE Europe

We compiled, from all available sources (Suppl. material 4: Table S4.2), a total of 14 occurrences of *M. sculpturalis* from the region most adjacent to the focal study area (Belgrade) – N Serbia and E Hungary (i.e. the eastern Pannonian Region), spanning the period of 2015–2019. Furthermore, we aimed to consider the spatio-temporal correspondence of eastern Pannonian records with the nearest records towards the west (i.e. towards the introduction core of Europe): we included 11 most adjacent records that are very broadly marginal to the wider Pannonian periphery: from E Austria, Slovenia and SW Croatia (2016–2019). Additionally, we considered the rare documented occurrences east of Serbia: one record from Romania (2019) and two records from the Crimean Peninsula (2018–2019). Therefore, we have covered, in a very broad sense, the area of SE Europe, wherein the colonisation by this species was documented only since 2015. Noteworthy, most records from Hungary after 2015 were discovered through a tailored web data extraction within the nationally based internet sources (previously being poorly accessible due to a language barrier). Findings from Serbia also became available with a delay; in the case of the record from Palić of 2018 (northernmost Serbia), it was due to a misidentification (at: Insekti Srbije 2018; corrected in 2020 by JBD).

The compilation and mapping of records were conducted within a more extensive Europe-wide survey of *M. sculpturalis* distribution and expansion; preliminary results for the period of 2008–2019 were presented as series of summary phase-maps in Ćetković et al. (2020) (available at: https://srbee.bio.bg.ac.rs/english). We herewith include a somewhat modified version of the summary map for 2019 (Suppl. material 1), with the regional records clearly delimited within the European distribution. All relevant details – data and metadata (coordinates, dates, bionomics, sources) used for this regional mini-survey are available in Suppl. material 4: Table S4.1. Records were georeferenced using the combination of Google Earth Pro ver. 7.3.3.7786 (Google Inc. 2020) and QGIS ver. 3.4 (QGIS Development Team 2018). Maps were made primarily with QGIS and the output images were further processed with various picture-editing software. We used the "Ruler" tool in Google Earth Pro to measure the linear distances between various adjacent occurrences (within and between the years in relation to the sequence of their detections), in order to quantify the basic spatial elements of apparent dispersal outcomes.

Results

Local scale: the Belgrade survey

Following the first detection of *M. sculpturalis* in Belgrade (and Serbia), in July 2017, we confirmed the establishment of this species only in August 2019. Our recording was almost exclusively based on bees foraging on *S. japonicum* trees. The exceptions were the first detected specimen – a male collected on *Trifolium repens* and a single female observed around a *Buddleja* bush; both cases occurred in downtown parks with nearby present *S. japonicum* trees. We did not detect *M. sculpturalis* neither on *Lavandula* nor on *Ballota* during the 2017–2019 period, despite notable efforts.

Most of the metrics calculated within the S500 framework had non-significant values (see in Suppl. material 5); therefore, we herewith present only the results from the S250 framework. Throughout the Belgrade area, we recorded *M. sculpturalis* at most locations where the current floral resource (CFR) of *S. japonicum* was sufficient to attract foraging bees at the moment of survey (Fig. 3A, B). The minimal sufficient value was CFR ≥ 0.1, found in 16 of 40 sectors (40%). Bees were recorded in 14 of 16 suitable sectors (88%); within five locations, we also collected specimens (22 females, 3 males). The estimated activity density of bees per sector ranged from 0 to 15.5 BpM (mean 4.66 ± 5.35 SD). The remaining sectors were recorded as without any blooming in 2019 or with blooming being already finished before our survey; hence, without possibility to detect bees (sectors with values for NoT_iB, TFR or CFR less than 0.05; see in Suppl. material 3). Of all tested metrics, only CFR had a significant effect on BpM (Table 1, Fig. 4A); when the values were averaged for each urbanistic zone, only CFR and %CFR had a significant effect on BpM (Table 2, Fig. 4B). We did not find significant differences in bee activity density amongst different urbanistic zones of Belgrade (H(4) = 4.521, p-value = 0.341).

Table 1. Results of the GLS linear regression models of the relationship of bee activity density (BpM) and variables NoT, NoT_iB, TFR and CFR (N = 16).

Model		Estimate	SE	t-value	p-value
NoT	Intercept	6.368	1.638	3.887	**0.002***
	Variable	-0.144	0.854	-1.644	0.122
NoT_iB	Intercept	6.092	2.001	3.045	**0.008***
	Variable	-0.557	0.579	-0.962	0.352
TFR	Intercept	3.459	2.689	1.286	0.219
	Variable	0.951	1.824	0.521	0.611
CFR	Intercept	-0.154	1.858	-0.089	0.935
	Variable	12.276	3.891	3.154	**0.007***

Significant p-values in bold (* p ≤ 0.01). NoT– number of trees; NoT_iB – number of trees in bloom; TFR – total floral resource; CFR – current floral resource.

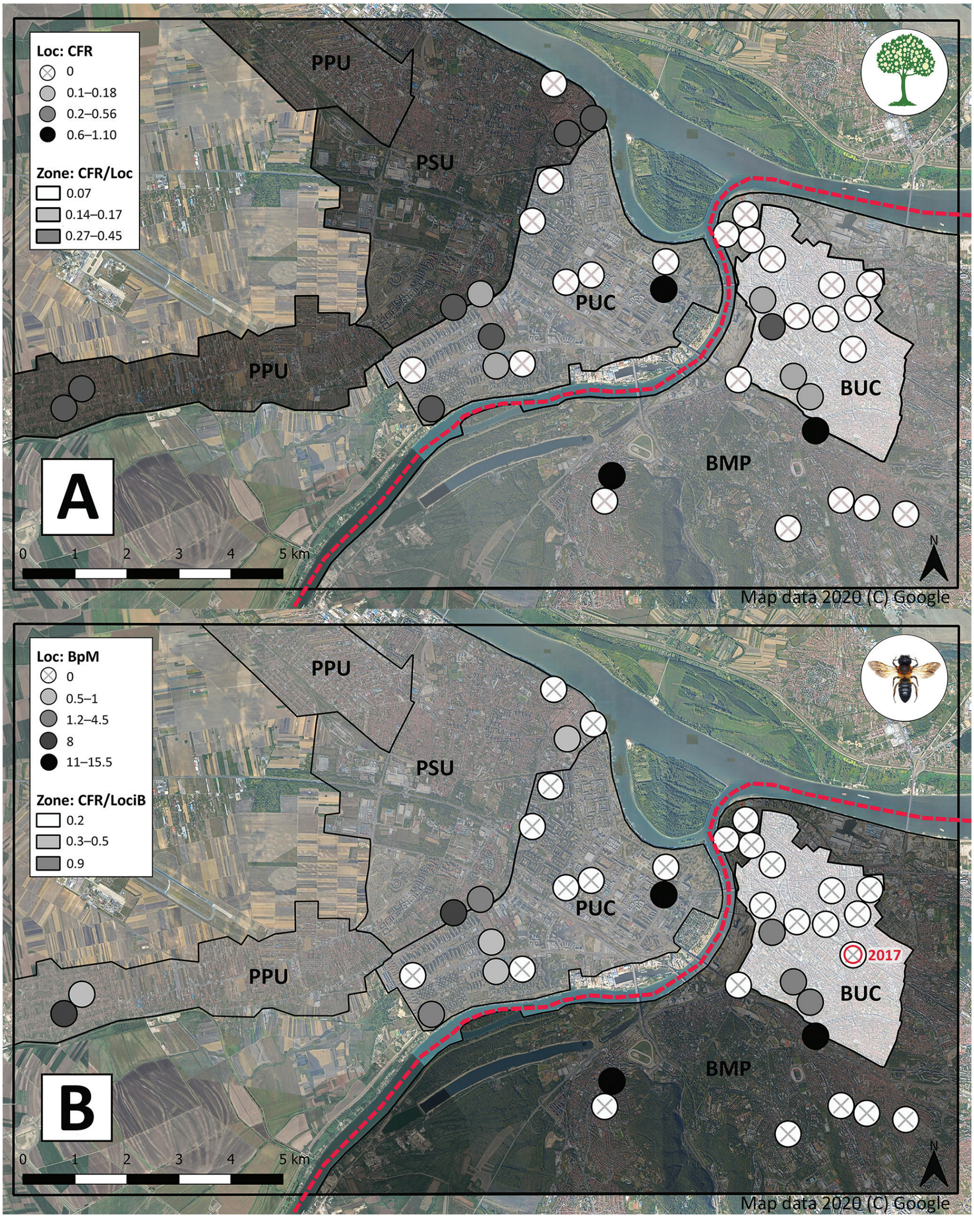

Figure 3. Distribution of **A** effective floral resources of *S. japonicum*, as surveyed in August 2019 (Current Floral Resource – CFR) and **B** respective metrics of *M. sculpturalis* activity density (Bees per Minute – BpM), both presented within the S250 framework (circular sectors – "landscapes" of r = 250 m; values shown in classes). Urbanistic zones (acronyms as in Fig. 1) are shown as background shades of grey, representing the averaged value of CFR per zone calculated either for **A** all 40 sectors or **B** only for 16 sectors with CFR ≥ 0.1. The location of the first find is marked with "2017". Numerical data available in Suppl. material 3: Tables S3.2–S3.4; see also maps in Suppl. material 5 for the complete visualisation of floral resource metrics.

Table 2. Results of the GLS linear regression models of the relationship of bee activity density (BpM) and variables TFR, CFR, %TFR and %CFR, all averaged across each urbanistic zone (N = 5).

Model		Estimate	SE	t-value	p-value
TFR	Intercept	1.575	5.663	0.278	0.799
	Variable	3.121	4.184	0.745	0.509
%TFR	Intercept	0.568	2.388	0.238	0.827
	Variable	15.359	6.196	2.479	0.089
CFR	Intercept	-2.492	0.909	-2.741	0.071
	Variable	18.008	1.838	9.798	**0.002***
%CFR	Intercept	1.293	0.756	1.711	0.186
	Variable	30.223	3.981	7.592	**0.005***

Significant p-values in bold (* p ≤ 0.01). TFR – total floral resource; %TFR – percentage of total floral resource; CFR – current floral resource; %CFR – percentage of current floral resource.

Within the surveyed area (16×9 km was the approximate span of all visited *S. japonicum* locations; Fig. 3A), we covered all urbanistic zones, with a varying number of surveyed locations between the zones (2–11; see also Suppl. material 2: Fig. S2.3). We counted the total of 490 *S. japonicum* trees (NoT), distributed quite unevenly across the study area (1–64 per sector). In 17 sectors (comprising 196 trees), we recorded no sign of blooming during 2019. Within the remaining 23 sectors, only on 51 trees we recorded at least some level of blooming in 2019 (NoT_iB; 12.2% of the total NoT). These blooming trees had a variable share of crowns effectively in bloom (TFR; 48.4% of the total NoT_iB); expressed per sector, TFR values ranged from 0.2 to 3.0 amongst these 23 sectors. As a reference high value, we established that the long-term average intensity of *S. japonicum* blooming in good seasons is at least ≥ 85% of the total crown volume (based on our observations from the seasons of 2017–2018 and 2020, which included about 60 and 550 individual trees, respectively). Accordingly, the sum of detected TFR available to bees during the summer of 2019 represented at most 5.9% of average *S. japonicum* resource availability in good seasons. At the time of our survey (2–9 August, the extent of available resources (CFR) was further reduced: only about 1.5% of the summed crown volume was still in bloom. The effective floral

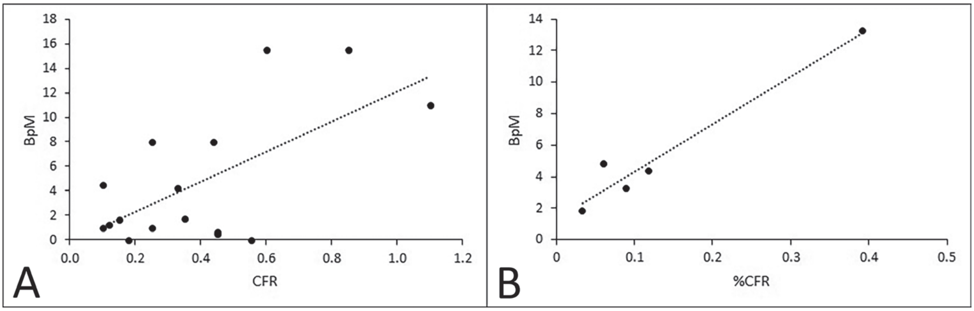

Figure 4. Relationship between **A** BpM and CFR and **B** BpM and %CFR averaged across each urbanistic zone (BpM – Bees Per Minute; CFR – Current Floral Resource; %CFR – percentage of current floral resource).

resources in early August (i.e. values of CFR ≥ 0.1) were recorded in only 16 sectors (totalling about 30% of the respective TFR summed value). Effective CFR values ranged from 0.1 to 1.1 per sector (Fig. 3A). Further details of all metrics are available in Suppl. material 3: Table S3.2, Table S3.3. For the spatial visualisation of established raw patterns, we presented the distribution of all four aspects of resource availability and respective *M. sculpturalis* activity density metrics, in a sequence of maps (Suppl. material 5: Figs S5.1–S5.2). We found no statistically significant differences in any of the floral resource metrics (NoT, NoT_iB, TFR, CFR) between different urbanistic zones (Suppl. material 5: Table S5.1).

Regional context: introduction and expansion within SE Europe

The first record in Serbia (in 2017, in Belgrade) was amongst the earliest known, positioned so remotely to the east from the contemporary colonised areas in western Europe. By that time, the closest previous occurrences were from NE Hungary in 2015 (Kovács 2015; ca. 330 km linear distance to the north) and from NW Slovenia in 2016 (Gogala and Zadravec 2018; ca. 550 km to the west). The closest contemporary occurrence was the first record in NE Austria (Westrich 2017; ca. 490 km northwest of Belgrade). With additional records in 2018, the apparent distribution gap across the eastern Pannonian Plain was reduced to ca. 160 km (from Belgrade northwards to Palić and Szeged). Additional adjacent records to the west (Austria, Slovenia and Croatia) remained at a fairly great distance throughout 2018–2019 (≥ 440 km). Detections further east in Europe (2018–2019) were more distant: ca. 1,000–1,150 km between Crimea (2018) and the closest records in Hungary (2015–2018) or Serbia (2017–2018); ca. 450–530 km between records in Serbia and Romania (2019); ca. 470–510 km between records in Hungary and Romania; ca. 640 km between records in Crimea and Romania. Gaps between the adjacent findings within the E Pannonian Plain were further reduced by the end of the 2019 season (ranging mostly 80–105 km, rarely 115–130 km, but in some areas only ca. 30–40 km), seemingly approaching a near-continuous distribution (cf. summary map in Suppl. material 1; details available in: Suppl. material 4: Table S4.1). Noteworthy, many records from this region were from the nesting situations, while none was from the proven pollen-source plants.

Discussion

We documented and analysed the widespread local occurrence of *M. sculpturalis* within the City of Belgrade, highlighting the early phase of its establishment in Serbia (2017–2019). This initially local case study provided a novel quantitative approach for assessing the bee activity in relation to floral resource availability, contributing to the framework for its early detection. Improved understanding of *M. sculpturalis* dynamic local patterns triggered an extension of the research scope to the wider, regional-scale context of this introduction – the colonisation within the E Pannonian Plain and SE

Europe (2015–2019). The combined outcomes of two complementary approaches, one on local and another on regional scale, provide important elements for future monitoring protocols of this Asian bee.

Local scale: the Belgrade survey

Detection and monitoring of a newly-established species may be challenging before a substantial local population build-up is attained (Hui and Richardson 2017), commonly involving a variously induced time lag after the initial introduction (Crooks 2005). We confirmed the presence of the sculptured resin bee at numerous locations throughout Belgrade in 2019, only two years after its first detection in Serbia. We suggest that such an early and widespread detection was enabled through the effect of "concentration" of bee foraging activities on a limited amount of the preferred floral resource. Namely, the summer of 2019 was characterised by an exceptional reduction of the bee's key food resource (*S. japonicum*): less than 13% of individual trees entered some level of blooming and only about 6% of the potential "blooming volume" was actually in bloom (TFR; as compared with good-blooming years); moreover, the availability of floral resources was further reduced during the short period of our survey (to 1.5%). Therefore, the average bee foraging intensity was concentrated by the factor of nearly 67 (i.e. it was 67 times more likely to observe active bees on inflorescences). Consequently, recording was highly successful: we detected *M. sculpturalis* in 88% of sectors in which the blooming of *S. japonicum* was sufficient to support at least the minimal bee foraging (the threshold value CFR ≥ 0.1 for this study design). The concentration effect may be particularly emphasised when a poor-blooming year follows a good year(s). This is based on a more general mechanism: alternating inter-annual fluctuations of blooming intensity of food plants may promote phase-delayed good or poor reproduction success of affected bee species (Tepedino and Stanton 1981; Crone 2013). Phase-delays produce a mismatch between the actual floral resources and the contemporary bee activity density and, in turn, the alternation of "concentration" and "dilution" effects. Blooming of *S. japonicum* seemingly follows a sort of alternating, but basically more irregular bearing pattern, a phenomenon otherwise well known in numerous tree taxa belonging to widely different plant families (Monselise and Goldschmidt 1982). The good blooming phase of *S. japonicum* in Belgrade during the first two years of *M. sculpturalis* documented presence (2017–2018) was favourable for the establishment and initial population build-up, albeit being slow. However, in the same period, its apparent activity has been diluted over this hyper-abundant and widely available floral resource, making it difficult to detect. We expect that observable activity density of *M. sculpturalis* remains decisively affected by this interplay of concentration and dilution phases, until a substantially abundant local population is attained. The preliminary outcomes from our 2020 survey (reduced recording success in conditions of a good-blooming season) are concordant with this expectation (Bila Dubaić et al. 2021).

Within the sectors with detectable bee activity (CFR ≥ 0.1), we have found that the activity density (BpM) was solely affected and significantly related to the levels of currently available floral resources (CFR); this was shown at both sector/landscape scale and as averaged values across urbanistic zones defined in this study. We could neither detect any effects of other tested resource parameters (NoT, NoT_iB, TFR) on bee abundance and distribution patterns, nor of other possible environmental features that vary amongst the defined urbanistic zones. Arguably, the lack of significant effects may be, in part, ascribed to a high variability of key floral resources and/or to a small sample size (due to the limited surveying period). However, this may also indicate the ability of *M. sculpturalis* to efficiently trace available key food resources, owing to its size and expectedly strong flight capacity (Quaranta et al. 2014; Westrich et al. 2015). Accordingly, it might be able to quickly optimise its foraging over sizable distances at the local scale, which is of particular importance when resources become critically restricted. Probably for the same reasons, our analysis has shown that a coarser-scale framework (S500) was less meaningful than the finer-scale one (S250), which seems counter-intuitive for such a large bee and, hence, worthy of further testing. Upon *M. sculpturalis* reaching higher, more stable abundances, it will be of interest to examine if other aspects of urban environmental gradients might also affect its local distribution and activity patterns (in addition to the key food availability). Of various features of urban environments, commonly emphasised as affecting wild bee diversity and/or dynamics (Hernandez et al. 2009; Fortel et al. 2014; Fischer et al. 2016; Leong et al. 2016; Baldock 2020), we expect that just a few might be proven as effective predictors of local differences in dynamic distribution patterns of *M. sculpturalis*. Probably the most relevant are features associated with gradients of urban temperature regimes – including heat island effects and associated shifts in local phenology of relevant plants. The bee phenology and the seasonal availability of food plants (either those foraged for pollen or as nectar sources) might be further modified by management regimes (watering, pruning etc.) of different urban settings. The main purpose of capturing such local differences – if shown significant – is to enable an accurate, while also feasible and rational framework for future monitoring schemes, i.e. for designing an appropriately stratified sampling (allowing for the minimal time investment etc.).

Noteworthy, even under dramatically reduced foraging opportunities on *S. japonicum* as the preferred food-plant, we could not detect the bee activity on alternative plants within the area. One such commonly available plant, *Lavandula*, is very frequently visited in the bee European range, second only to *S. japonicum* (cf. Ćetković et al. 2020: extracts from the ongoing study). In some country accounts, such as France (Le Féon et al. 2018) and Italy (Ruzzier et al. 2020), *Lavandula* was even ranked as first (based on all available records); however, more frequent casual encounters of *M. sculpturalis* in southern France and northern Italy became common only > 8 years upon respective first detections. Such a difference in visitation patterns between western Europe and Serbia was likely affected by a higher population abundance in areas where *M. sculpturalis* persisted for longer time periods. Its higher abundances could have promoted a spill-over effect of surplus bees, which were more easily attracted to other available plants, at least for nectar (*Lavandula* and *Buddleja* are probably not suitable as pollen source – cf. Ćetković et al. 2020). Conversely, the lack of records on other plants in the Belgrade area may be indicative of the local bee population not yet reaching the level of abundance that could support spill-over effects.

Understanding of genuine plant usage patterns is important for improving *M. sculpturalis* early detectability, as well as for further monitoring of its population trends. The effect of concentration, herewith based specifically on a single key food plant, was crucial for this early mass recording. Without this effect, the initially slow population growth would translate into a prolonged accumulation of rare accidental records. For this reason, species detection in many areas commonly lags behind its actual establishment and expansion. Such detection patterns are documented elsewhere in Europe (cf. Le Féon et al. 2018; Lanner et al. 2020a; Ruzzier et al. 2020 etc.), but without any consideration of possible mechanisms behind these time lags (cf. Crooks 2005). In turn, our results further emphasise the relevance of *S. japonicum* as the single most important food plant for establishment and spreading of the bee, as well as for its efficient recording, at least when bee population levels are low. Despite quite numerous plant taxa listed in various treatises of bee-plant interactions, affiliation of *M. sculpturalis* with selected members of the Fabaceae plant family seems by far the most relevant for pollen provisioning (Ćetković at al. 2020; see also relevant references in Introduction). Furthermore, *S. japonicum* is the only widely available, mass-blooming and phenologically suitable representative of the large-flowered Fabaceae in the Belgrade area and a similar situation exists in many Serbian cities and towns. Thus, to enable the early detection and to improve the efficiency of surveillance efforts in areas of suspected bee presence (or expected arrival), attention should be focused on locations with easily accessible, but not excessively abundant and too widely dispersed, key plant resources. Most suitable test-locations might be small towns or villages with preferably just a few *S. japonicum* trees, surrounded by wider landscapes that are poor in any proven pollen-source plants. These situations might correspond with effects of concentration, documented herewith for Belgrade in 2019. However, a suitable approach is yet to be conceived for assessing the eventual spreading of *M. sculpturalis* through vast semi-natural or wilderness areas.

Several studies urged for the establishment of monitoring programmes to track the expansion and evaluate possible impacts of this rapidly spreading alien bee (Quaranta et al. 2014; Le Féon et al. 2018; IUCN 2020; Ruzzier et al. 2020; Ribas Marquès and Díaz Calafat 2021). So far, comprehensive studies in colonised regions of Europe were more extensively based on opportunistic recordings of nesting activity (within artificial or natural settings), often with a substantial involvement of citizen scientists or through casual/scattered public contributions to various internet-based data repositories (Le Féon et al. 2018; Lanner et al. 2020a, 2020b; Ruzzier et al. 2020; Westrich 2020). Nesting-based monitoring may be organised as spatially effective, providing that a sufficiently wide network of voluntary observers could be organised and motivated to install tailored nesting facilities around their homes/workplaces, to regularly observe various bee activities and to tediously document and report their recordings. However, this approach may not be uniformly feasible across Europe, due to regionally variable citizen's attitudes or prior experiences (Pocock et al. 2018; Requier et al. 2020). Furthermore, it is possibly not best suited for the early phase of colonisation, due to the likely poor effectiveness in recording a too sparse bee activity density (i.e. poor effort-efficiency ratio). Therefore, it should be regarded as complementary to active and field-intensive surveying of focal plants and bee activities on flowers. Undoubtedly, the combination of both approaches will be needed for the evaluation of potential invasiveness of this first widespread alien bee in Europe.

Currently, we still lack an elaborate and comprehensive monitoring protocol – generally for any of the alien bee species worldwide. In this study, we propose a set of surveying routines and analytical approaches suitable for a structured assessment of plant resource availability, integrated with a standardised quantification of sculptured resin bee activity density. To build a functional monitoring approach, this working framework requires further testing and quantitative "calibration" of suggested procedures, under different environmental settings and varied modalities specific for each local or regional colonisation event. This should be based on extensive comparison of future assessment trials, taking into account the complicated interplay of resources: the co-occurrence of favourable plants (of different functional status: pollen or nectar-only sources), their varying phenologies and management regimes at different scales (from landscape through to regional), affected by varying environmental gradients (from urban to natural), while also considering particular establishment histories.

Regional context: introduction and expansion within SE Europe

The first three occurrences of *M. sculpturalis* east of the Alps, as documented during 2015–2017 (NE Hungary, N Serbia and NE Austria), were remarkably distant from the contemporary W European range and also widely mutually separated across the Pannonian Plain (Suppl. material 1). Accordingly, all were considered as likely cases of long-distance jump dispersal (Kovács 2015; Ćetković and Plećaš 2017; Lanner et al. 2020a), relative to a largely continuous range expansion within W Europe. Further to the east, the position of the sole record in Romania (from 2019) matches the relative distances of dispersal events of 2015–2017, while the dispersal jump to Crimea (in 2018) was outstandingly long-distant. Therefore, the overall pattern of this "SE European phase" of *M. sculpturalis* expansion appears as surprisingly different from the dispersal history in W Europe during 2008–2019 (cf. Ćetković et al. 2020; Suppl. material 1). Herewith, we consider the elements of alternative regional introduction and expansion scenarios.

From this wider perspective, a long-distance jump into Belgrade indeed seems as the most plausible scenario. The status of Belgrade (the capital city) and its position at important traffic junctions of several major routes from central and western Europe, makes it highly exposed to a large-scale transportation of diverse goods (Suppl. material 2: Fig. S2.1). The lack of records from most of Serbia and also from most of neighbouring countries, might further support the hypothesis that Belgrade was the genuine introduction point for Serbia (and for the Central Balkans). However, the initial dispersal distances of elaborated SE European cases do not allow for more specific inferences regarding the origin. Generally, human-aided secondary introductions amongst the recently established, but widely isolated locations within SE Europe are not likely, since the initial low-abundances reduce the chances for inadvertent passive transportations (Bertelsmeier and Keller 2018). Therefore, the source(s) of these presumed long-distance jumps within SE Europe could have been any population from the earlier-established W European range; even the overseas origins cannot be excluded (Kovács 2015). The recent estimates of genetic relatedness suggest that the introduction into NE Austria represents an independent colonisation event in Europe, i.e. not originating from populations established in France and Switzerland (Lanner et al. 2021).

However, an in-depth consideration of two contrasting cases (Belgrade vs. E Pannonian) suggests that the alternative scenario of the colonisation of N Serbia is even more plausible; it is based primarily on a diffusive mode of spreading (Suarez et al. 2001). The vivid nesting activity of *M. sculpturalis* in the small Hungarian town of Gyöngyös (Kovács 2015) indicates that local establishment has happened in one or more seasons before the actual detection. Its likely longer and more extended presence in the NE Pannonian Region is further emphasised with predominance of nesting-based records over the plant-based ones in reports from 2018. The seemingly abrupt expansion of its apparent range across the NE Pannonian Plain in 2018, only three years after the first detection, cannot be based on further human-assisted jump dispersal events. More likely, a slow "sneaking" diffusive dispersal was taking place almost continuously for several years, probably for a much longer period than could be inferred from the available recordings. Accordingly, before more substantial abundances could become obvious (simultaneously throughout the region), the southward spreading across the Pannonian lowlands could have already reached the northern Balkans (i.e. Serbia in 2017), without being detected in the intermediary area before 2018. Therefore, the impression of a genuine, fairly distant jump into Belgrade, unrelated to the prior introduction in NE Hungary, is most probably an artifact, i.e. the "type III" lag phase (Hui and Richardson 2017, after Crooks 2005). Somewhat contrasting evidence of *M. sculpturalis* spreading patterns at two analysed spatio-temporal scales (local vs. regional) indicates that it lacks the true lag phase (i.e. the "type II" of Hui and Richardson 2017). The usually slow initial population build-up apparently does not hamper the active and successful spreading of this bee, but coupled with a relatively scattered faunistic research in the area, it resulted in a poor detection in the region during at least three years (since 2015). Noteworthy, the widespread presence (since 2018) was documented merely through accidental/casual activity of citizen scientists (Rovarok, pókok 2017–2019; Insekti Srbije 2018; izeltlabuak.hu 2018), i.e. without any focused research.

The recognition of one vs. another mode of dispersal, as well as the identification of a probable introduction and expansion pathway(s), may be severely difficult and often speculative, but nevertheless highly important for understanding the spatio-temporal patterns of each non-native colonisation (Suarez et al. 2001; Trakhtenbrot et al. 2005; Hui and Richardson 2017). Herewith, we contrasted the evidence from methodologically different approaches (focused/systematic surveillance, based on focal plant resources and casual/opportunistic recording through unfocused citizen observations) at two similar temporal scales (3 vs. 5+ years), but over largely different spatial scales (< 20 km vs. > 300 km). The study revealed somewhat contrasting, but complementary expansion and detection patterns, as important aspects of usually hidden early colonisation dynamics, which are of great methodological relevance for future monitoring. We suggest that, in the case of the bee with relatively narrow and well-established trophic requirements, focusing on key floral resources and concentration-dilution effects is a highly profitable approach. Nevertheless, the evidence lacking this component may be highly useful in reconstruction of expansion modes and pathways, if interpreted within a suitable spatio-temporal framework and well-understood recording context. The source of *M. sculpturalis* SE European introduction(s) would be more conclusively identified with a molecular genetic approach (Bila Dubaić and Lanner 2021; Lanner et al. 2021), which will provide a better understanding of dispersal modes and general pathways. However, fine-scale exploratory studies focused on revealing the regional expansion dynamics and patterns of local interactions will remain a highly relevant approach for establishing efficient detection and surveillance.

Finally, we have shown that, contrary to common expectations (Quaranta et al. 2014; Lanner et al. 2020a), the striking appearance and easy to observe behaviour of *M. sculpturalis* is not sufficient to ensure the very early detection and real-time tracking, without a specifically tailored surveillance approach. However, this bee represents a highly suitable and prospective model organism for comprehensive studies of non-native bee colonisations.

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Supplementary material 1

Megachile sculpturalis distribution through Europe for the period 2011–2019

Authors: Jovana Bila Dubaić, Milan Plećaš, Jovana Raičević, Julia Lanner, Aleksandar Ćetković

Data type: map (.pdf file)

Explanation note: Summary visualisation of the *Megachile sculpturalis* distribution and spreading through Europe for the period 2011–2019, shown as series of tentative expansion phases.

Link: https://doi.org/10.3897/neobiota.73.80343.suppl1

Supplementary material 2

Study area – Belgrade (Serbia): basic topography, biogeography, ecological patterns and urbanistic zonation

Authors: Jovana Bila Dubaić, Milan Plećaš, Jovana Raičević, Julia Lanner, Aleksandar Ćetković

Data type: maps (.pdf file)

Explanation note: Study area – Belgrade (Serbia): basic topography, biogeography, ecological patterns (habitats, land-use, landscapes) and urbanistic zonation: (i) City of Belgrade: general features (Fig. S2.1); (ii) Zonation of Belgrade (version_01: survey in 2019; Fig. S2.2); (iii) Survey design and processing of geospatial framework (Fig. S2.3) (This is the PDF version of selected pages from the thematic project website (Ćetković et al. 2020), by: Centre for Bee Research of the Faculty of Biology, University of Belgrade (available also at: https://srbee.bio.bg.ac.rs/english/belgrade-general-features; https://srbee.bio.bg.ac.rs/english/m-sculpturalis-2019-survey; with occasional updates).

Link: https://doi.org/10.3897/neobiota.73.80343.suppl2

Supplementary material 3

Quantitative survey of distribution and abundance parameters of *M. sculpturalis* and *S. japonicum* in the Belgrade area in August 2019

Authors: Jovana Bila Dubaić, Milan Plećaš, Jovana Raičević, Julia Lanner, Aleksandar Ćetković

Data type: spreadsheet database (excel file)

Explanation note: Quantitative survey of distribution and abundance parameters of *M. sculpturalis* (BpM) and *S. japonicum* (NoT, NoT_iB, TFR, CFR) in the Belgrade area in August 2019: Tables S3.1–S3.4.

Link: https://doi.org/10.3897/neobiota.73.80343.suppl3

Supplementary material 4

Records of *M. sculpturalis* from the broader SE European region (compiled for: 2015–2019)

Authors: Jovana Bila Dubaić, Milan Plećaš, Jovana Raičević, Julia Lanner, Aleksandar Ćetković

Data type: database (excel file)

Explanation note: Table S4.1. Records of *M. sculpturalis* from the broader SE European region and the adjacent areas (compiled for: 2015–2019). Table S4.2. Published data sources used.

Link: https://doi.org/10.3897/neobiota.73.80343.suppl4

Supplementary material 5

Results of statistical testing and distribution maps of estimated metrics

Authors: Jovana Bila Dubaić, Milan Plećaš, Jovana Raičević, Julia Lanner, Aleksandar Ćetković

Data type: maps (.pdf file)

Explanation note: Belgrade area: results of statistical testing (Tables S5.1–S5.3) and distribution maps of estimated metrics (Figs S5.1–S5.2).

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Interaction effects of desiccation and temperature stress resistance across *Spodoptera frugiperda* (Lepidoptera, Noctuidae) developmental stages

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Abstract

Insects encounter multiple overlapping physiologically challenging environmental stressors in their habitats. As such, the ability of insects to withstand these stressors singly or interactively is fundamental in population persistence. Following its invasion in Africa, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) has successfully established and spread in most parts of the continent. However, the mechanisms behind its successful survival across arid and semi-arid African environments are relatively unknown. Here, we investigated the water balance of *S. frugiperda* across its developmental stages. Given the relationships between desiccation stress, temperature stress and other life history traits in arid ecosystems, we also measured interaction effects across metrics of these traits. Specifically, we measured basal body water content (BWC), water loss rates (WLRs) and the effects of desiccation pre-treatment on critical thermal minimum (CT_{min}), critical thermal maximum (CT_{max}) and fecundity. Body water content and WLR increased with age across larval instars. However, the effects of desiccation environments on WLRs were more dramatic for 5th and 6th larval instars. The 5th and 6th instars exhibited highest BWC and magnitude of WLRs plastic responses following desiccation treatment. The effects of desiccation pre-treatment on temperature tolerance were less apparent, only significantly improving CT_{min} in 2nd and 3rd larval instars and reducing CT_{max} in 5th instars. In addition, desiccation pre-treatment showed no significant effects on fecundity. These results show that water balance traits differ with developmental stage, while the effects of desiccation pre-treatment were more dramatic and inconclusive. The differential desiccation resistance, high proportional BWC and no desiccation pre-treatment effects on fecundity may help the species survive in arid and semi-arid environments. This information provides insights into understanding *S. frugiperda* survival under desiccating arid and semi-arid tropical environments and is significant in predicting pest outbreaks.

Keywords
Desiccation pre-treatment, fall armyworm, fecundity, thermal plasticity, water balance

Introduction

In natural and managed ecosystems, insects encounter multiple overlapping environmental stressors which may affect their physiological fitness and survival (Gotcha et al. 2018; Mutamiswa et al. 2018, 2021). For example, there is a higher likelihood of increasing magnitudes and frequency of heat and desiccation stress as well as drought (associated with poor rainfall) as climate change ensues (IPCC 2014). As such, the interplay between heat stress and water deficit may exert physiological stress amongst insects and across ontogeny, and in all likelihood affecting organismal fitness, function and survival (Bubliy et al. 2012; Mitchell et al. 2017). Given that rapid dehydration occurs under dry environmental conditions, prolonged exposure to these conditions may negatively influence insect water balance and homeostasis (Guedes et al. 2015). Hence, survival may be highly dependent on body water content (BWC) management and desiccation tolerance. Various studies suggest that insects from tropical and drier environments have inherent adaptive water balance mechanisms such as reduction in cuticular permeability, reduced water loss rates (WLRs) through excretion and differential cuticular lipids composition and plasticity thereof (Hadley 1994; Gibbs et al. 1997; Gibbs 2002; Hoffmann et al. 2003a; Kellermann et al. 2009). Most empirical studies looking into the effects of insect responses to climate change have been skewed towards high temperature responses (Huey and Kearney 2020). However, precipitation and relative humidity (RH) fluctuations also exert significant stress to insects and tolerance to these stressors can largely affect species ecology (Chown et al. 2011; Kellermann et al. 2018). Moreover, increasing heat stress with climate change (see Stillman 2019; Xu et al. 2020) also comes with simultaneous high desiccation stress. Therefore, understanding water balance and heat stress, and the interactive responses of the two related stressors may be significant for survival in arid and semi-arid habitats and may be key determinants to invasion success for pest insect species (Kellermann et al. 2018).

Water balance is the ability of an organism to maintain constant body water levels under different environments (Yoder et al. 2003). It plays a pivotal role in determining insect diurnal and seasonal activity patterns, population dynamics and biogeography (Kleynhans and Terblanche 2011; Guedes et al. 2015). The ability of an insect to maintain water balance (water gain and loss equilibrium) is highly dependent on the moisture content of its immediate environment (Guedes et al. 2015). Insects lose water through the exoskeleton, evaporation through spiracles and tracheal system, oral or anal secretions, and excretion (Chown et al. 2011). However, the bulk of the water is lost through the exoskeleton (Chown and Nicolson 2004; Rolandi et al. 2014). Given their small size, low fat-storage and a large surface area to volume ratio, maintenance of water balance in insects is a challenge, and significantly so in arid to semi-arid tropical changing environments (Gibbs et al. 1997; Gibbs et al. 2003; Chown et al. 2011; Weldon et al. 2013; Rolandi et al. 2014; Bujan et al. 2016). Insect water loss is also highly complex and varies with many factors including clines, species, environmental history (or plasticity) and ontogeny. However, the ontogenetic effects on water balance studies remain limited despite the significance of the traits for insects' population dynamics and biogeography. In contrast, insects gain water directly through drinking free water, indirectly through food and nectar as well as absorbing atmospheric water (Chown and Nicolson 2004; Guedes et al. 2015). As a result, the maintenance of hygric physiological homeostasis may be a key trait for survival and coupled with heat stress resistance, the two traits may be critical for surviving high temperature and desiccation in arid environments, typified with climate change. However, despite the significance of these two overlapping stressors, their interactive effects across ontogeny remain unknown for *Spodoptera frugiperda* (J.E Smith) (Lepidoptera: Noctuidae), despite its significance for the pest species ecology.

Desiccation stress, commonly associated with xeric arid environments is one of the primary stressors influencing the distribution and behavior of insects in the tropics. As insects move from moister to drier environments, there is a high likelihood of experiencing increasingly desiccating conditions (Weldon et al. 2016). As a result, some organisms are spatially restricted to areas of optimal RH, or behaviorally track optimal RH conditions. For example, *Drosophila birchii* is usually restricted to rainforests due to its inability to tolerate desiccation stress (Hoffmann et al. 2003b; Bazinet et al. 2010). Hence, desiccation resistance (the ability of insects to withstand water loss) is fundamental in managing stress under rapidly shifting environments and can largely predict the fundamental niche of invasive pest species (IPCC 2014; Bujan et al. 2016). Desiccation resistance varies across geographic clines, body mass and biochemical composition (Guedes et al. 2015; Bujan et al. 2016), size and sex (Tejeda et al. 2014), age (Weldon and Taylor 2010), life stage and species (Weldon et al. 2013, 2016; Bujan et al. 2016). Similarly, within species desiccation resistance also varies across individual populations and development (Weldon and Taylor 2010). Insects have evolved some behavioral, morphological, physiological and biochemical mechanisms to withstand dehydration, and these are primarily hinged on increasing water storage, restricting WLRs and increasing tolerance to water loss (Gibbs et al. 2003; Chown and Nicolson 2004; Chown et al. 2011; Weldon et al. 2013, 2016). Behaviorally, insects can migrate to cooler moister microhabitats, aggregate and burrow to reduce desiccation stress (Guedes et al. 2015). In addition, insects can physiologically compensate for desiccation stress by converting stored fat to metabolic water, increasing cryoprotectants levels, decreasing metabolic rate and increasing reabsorption of water in the rectum (Benoit et al. 2007; Lopez-Martinez et al. 2009; Harrison et al. 2012). Using *Anastrepha ludens* (Tejeda et al. 2014) and Panama ant species (Bujan et al. 2016), previous studies showed a significant correlation between desiccation resistance and body size owing to an increased BWC with body size. However, in *Bactrocera tryoni* (Weldon and Taylor 2010), desiccation resistance decreased with age and body size while for *Belgica antarctica*, the trait also varied across developmental stages (Benoit et al. 2007). This suggests complexity in determinants for desiccation stress resistance and warrants more investigations across different taxa before general conclusions can be made on desiccation resistance in insects.

Acclimation is a medium to long term, often reversible, change in phenotype in response to chronic exposure to sub-lethal stress under controlled conditions and within a single generation of an organism (Whitman and Agrawal 2009; Sgrò et al. 2016). This mechanism gives insects a performance advantage on a subsequent exposure to stress in the same environment. For example, insects exposed to prior stressful but sublethal dry environments tend to have lower WLRs on subsequent exposure to more severe desiccation stress (Terblanche and Kleynhans 2009; Chown et al. 2011). Various studies have pointed to the significant role desiccation acclimation plays in fitness and survival of different insect taxa, singly and interactively with other stressors e.g. temperature. For example, desiccation acclimation improved critical thermal minimum (CT_{min}) in larvae of *Chilo partellus* (Swinhoe), *Busseola fusca* (Fuller) and *Sesamia calamistis* Hampson (Mutamiswa et al. 2018). In addition, desiccation acclimation also increased heat tolerance (CT_{max}) in *Ceratitis rosa* Karsch adults (Gotcha et al. 2018) as well as larvae and adults of *Prostephanus truncatus* (Horn) (Mutamiswa et al. 2021). These studies suggest potential positive interactive effects across divergent stress traits, a phenomenon that may be key to maintaining fitness under stressful heterogeneous environments (see Sinclair et al. 2013).

Spodoptera frugiperda is a tropical insect of American origin which was first reported in Africa in 2016 (Goergen et al. 2016; Stokstad 2017). Since then, it has successfully invaded African countries (Stokstad 2017), Asia (FAO 2018a; Sharanabasappa et al. 2018; Yigezu and Wakgari 2020) and recently Australia (Maino et al. 2021). It is highly polyphagous (Cock et al. 2017; Montezano et al. 2018; Tambo et al. 2019) but it mainly prefers maize (Goergen et al. 2016; Abrahams et al. 2017; FAO 2018b). Its resilience and ability to survive all year round in African countries has been a topic of great concern (Early et al. 2018; Megersa and Tamiru 2019; Keosentse et al. 2021). Previous studies have assayed its thermal tolerance (Barfield et al. 1978; Du Plessis et al. 2020; Keosentse et al. 2021) and developed models to predict its spatio-temporal distribution (Westbrook et al. 2016; Early et al. 2018; Du Plessis et al. 2018). However, mechanisms behind its invasion success in tropical and arid environments remain poorly elucidated. The movement of *S. frugiperda* across humid and dry African environments, its ingression and naturalization may be better explained by efficient mechanisms of water balance, heat tolerance and their interactive effects thereof. Empirical studies on water balance have been reported in *Sitophilus zeamais* Motschulsky (Guedes et al. 2015), *Ceratitis capitata* (Wiedemann), *Ceratitis cosyra* (Walker) and *C. rosa* (Weldon et al. 2016). However, no studies have investigated developmental stage related differences in water balance traits of *S. frugiperda*. In addition, no studies have looked at the interaction of desiccation and high temperature stress for the species, despite being significant for fitness in dry tropical habitats. Against this background, we investigated developmental stage related differences in water balance in *S. frugiperda*. Furthermore, we also tested the effects of desiccation environments on the physiological traits *vis* WLRs and thermal tolerance and ecological traits, including effects on fecundity. Given *S. frugiperda*'s high invasion propensity under shifting environments, we hypothesize that desiccation pre-treatment capacity coupled with highly integral water balance may enhance its fitness in novel arid and semi-arid environments. We also hypothesize that there is variation in BWC, WLRs and thermal tolerance following acclimation across *S. frugiperda* developmental stages. The information generated here may help in modeling species distribution and developing management options under globally shifting environments.

Materials and methods

Insect culture and rearing conditions

Wild populations of *S. frugiperda* 1st instar neonate larvae from different cohorts of parents were collected from maize fields in Bobonong village (22.26195°S, 28.99985°E) in the Central district of Botswana during austral summer (November 2020 to March 2021). They were mass reared in the laboratory on artificial diet (see Tefera et al. 2019) under optimum conditions i.e. 28 ± 1 °C; 65 ± 5% RH; 12:12 light: dark photoperiod in climate controlled chambers (HPP 260; Memmert GmbH + Co.KG, Germany). Since cannibalism is predominant amongst late larval instars (Chapman et al. 1999), each late instar larva (3rd onwards) was individually placed in a separate 100 ml plastic vial with perforated screw-cap lid and artificial diet until pupation. Pupae were maintained in open Petri dishes in Bugdorm rearing cages (320 cm^3; Bugdorm-BD43030F, Megaview Science Co., Ltd, Taiwan) until adult eclosion. Following eclosion, adults were provided with 25% sugar water-soaked cotton wool in a 60 ml plastic vial for food *ad libitum*. At least two potted maize plants (2 weeks old) were placed in each rearing cage as oviposition substrate for gravid females. After hatching the 1st instar larvae were transferred to artificial diet for subsequent rearing. For all experiments, all larval instars, 24–48 h old pupae and adults (unsexed) F_1 populations were used. Larval instar stages were demarcated using head capsule size (see Montezano et al. 2019).

Body water- and proportional body water-content

Twenty insects from each larval instar (1–4) were individually placed in 0.5 ml microcentrifuge tubes of known weight while instars (5–6), pupae and moths were individually placed in 2 ml microcentrifuge tubes of known weight. The initial mass of each insect was measured (to 0.0001 g) on an analytical balance (AS220.R2, RADWAG, Poland). Thereafter, the insects were placed in a Memmert drying oven (UF160, Memmert, Germany) set at 60 °C for 48 hours. Dry mass was measured (to 0.0001 g) on a microbalance after allowing insects to cool under laboratory temperature (28 °C) for 15 minutes. Dry mass was subtracted from initial mass to determine initial BWC (see complete methods in Bazinet et al. 2010; Weldon et al. 2018). Proportional BWC, basal body water (g) divided by wet mass (g) was calculated and used for analyzing developmental stage effects for this trait.

Water loss rates

Larval instars, pupae and adults (N = 20 for each developmental stage) were individually placed in pre-weighed perforated 0.5 ml and 2 ml microcentrifuge tubes of known weight. The weight of each insect-carrying tube was later measured on an analytical microbalance. Thereafter, insect weight was determined by subtracting initial weight of tube from weight of insect-loaded tube. These tubes were placed on top of wire gauze in a glass sealed desiccator containing 80 g silica gel (equivalent to 7% RH) in climate chambers set at 28 °C; 65% RH for treatment while controls were placed in a desiccator without silica gel. After every 12 h, the insects were removed from each climate chamber, weighed in their tubes to calculate weight loss, and then immediately placed back in the chambers for subsequent recordings. This was repeated for 48 hours for all the developmental stages. Water loss rate (mg hr^{-1}) over the 48 h experimental duration was calculated as follows:

$$WLR = \frac{M1 - M2}{T}$$

where WLR = water loss rate, M_1 = initial body mass, M_2 = final body mass and T = time (hours) following methods by Weldon et al. (2016) and Mutamiswa et al. (2021).

Effects of desiccation pre-treatment on physiological (critical thermal limits [CTLs]) and ecological traits (adult fecundity)

Desiccation pre-treatment assays were carried out using standard established protocols (see Bauerfeind et al. 2014; Mutamiswa et al. 2018) before measuring physiological and ecological traits. *Spodoptera frugiperda* larval instars and adults were individually placed in ten perforated 0.5 ml and 2 ml microcentrifuge tubes. The tubes were placed on top of wire gauze in a glass sealed desiccator containing 80 g silica gel. Thereafter, the desiccator was placed in a climate chamber under optimum conditions for 24 h. Thermocron iButtons (Model DS1920; Dallas Semiconductors, Dallas, Texas) were used to measure temperature and RH inside the desiccator at 30-min intervals. After 24 h, the insects were transferred to artificial diet for a further 12 h under optimum conditions before measuring CTLs and fecundity. Control organisms were maintained in climate chambers at (28 °C; 65% RH) before measuring their CTLs and fecundity.

Critical thermal limits

Ten individual *S. frugiperda* larvae (of each 1–6th instars) and adults (mixed sex), from desiccation pre-treatment were placed into a double jacketed chamber ('organ pipes') connected to a programmable water bath (Lauda Eco Gold, Lauda DR.R. Wobser GMBH and Co. KG, Germany) filled with 1:1 water: propylene glycol to allow for subzero temperatures. A thermocouple (type K 36 SWG) connected to a digital thermometer (53/54IIB, Fluke Cooperation, USA) was inserted into the middle/control test tube to record chamber temperature. Both critical thermal- maxima (CT_{max}) and -minima (CT_{min}) experiments commenced at a set point temperature of 28 °C (with 10 minutes equilibration time) from which temperature was ramped up (CT_{max}) and down (CT_{min}) at a rate of 0.25 °C/min until their CTLs were reached. The experimental procedure was repeated twice for each trait and treatment (1–6th instars plus adults) to yield sample sizes of N = 20 for each treatment. Controls were maintained in climate chambers under optimum conditions (28 °C; 65% RH) before measuring their CTLs. Critical thermal limits were defined as the lower (CT_{min}) and upper (CT_{max}) temperatures at which each individual insect lost coordinated muscle function, consequently losing the ability to respond to mild stimuli (e.g. prodding) (see discussions in Nyamukondiwa and Terblanche 2010; Mutamiswa et al. 2017).

Fecundity

Twenty replicate pairs of treatment (desiccation pre-treated) 1–2 day old female and male adults of *S. frugiperda* were placed in separate rearing cages in climate chambers under optimum conditions. Each pair was provided with a cotton wad soaked in 25% sugar-water and a potted maize plant (2 weeks old) for oviposition. Control adult pairs (male and female) were maintained at optimal conditions (28 °C; 65% RH) until egg laying. Insects were allowed to mate, oviposit and removed from the experimental cages on day 8. Eggs were allowed to hatch on maize plants and emerging 1st instar neonate larvae were counted and removed from the cage immediately after hatching. Fecundity was defined as the number of emerging 1st instar neonate larvae per pair following adult oviposition for the 7 days duration.

Data analyses

Data analyses were carried out in STATISTICA, version 13.0 (Statsoft Inc., Tulsa, Oklahoma) and R version 3.3.0 (R development Core Team 2020). Data were first checked for normality and equality of variances using the Shapiro-Wilk and Hartley-Bartlett tests respectively. For BWC, proportional BWC, fecundity and CTLs data, linear model assumptions of constant variance and normal errors were met while WLR data failed to conform to assumptions of analysis of variance (ANOVA). Therefore, BWC, proportional BWC and fecundity results were analyzed using one-way ANOVA while CTLs results were analyzed using full factorial ANOVA with developmental stage and treatment being the categorical factors whereas BWC, proportional BWC, fecundity, CT_{min} and CT_{max} were the dependent variables. Water loss rates were analyzed using generalized linear model (GLM) assuming a Gaussian distribution and an identity link function in R with duration, developmental stage and time being the categorical factors and WLR being the dependent variable. Tukey-Kramer's *post-hoc* tests were used to separate statistically heterogeneous groups.

Results

Body water content

Body water content varied significantly across developmental stages ($P < 0.001$) (Table 1, Fig. 1A). The mean BWC for all larval instars (1st–6th), pupae and adults were 0.01±0.001 g, 0.03±0.002 g, 0.06±0.002 g, 0.11±0.005 g, 0.19±0.007 g, 0.29±0.01 g, 0.15±0.005 g and 0.05±0.002 g respectively (Fig. 1A). Body water content significantly increased with larval instar level with instar 1 recording the lowest BWC whilst the 6th instar recorded the highest BWC amongst all tested developmental stages (Fig. 1A). However, there was no significant difference in BWC between 1st and 2nd, 2nd and 3rd and across 2nd and 3rd larval instars and that of adults (see Fig. 1A). Proportional BWC also differed significantly among developmental stages ($P < 0.001$) (Table 1, Fig. 1B). Adults recorded the lowest proportional BWC (0.63±0.01), followed by pupae (0.71±0.005) while larvae recorded the highest proportional BWC, albeit it was not significantly different amongst all instars (Fig. 1B).

Water loss rate

Generally, WLRs seemed to increase with larval instar, consistent with the constituent BWC. Water loss rate significantly varied across developmental stages, and time ($P < 0.001$) and not significantly among treatments ($P > 0.01$) (Table 2, Fig. 2). For all tested developmental stages, there seemed to be no significant effect of desiccation environment on WLRs except for 5th and 6th larval instars (see Fig. 2), developmental stages that also recorded the highest BWC (Fig. 1A). These trends were consistent across the different times (12, 24, 36 and 48 h). Moreover, WLRs gradually decreased over time for instars 4, 5 and 6 as well as adults of *S. frugiperda*. First instar larvae recorded the lowest WLR while 6th instar larvae recorded the highest WLR across time (Fig. 2). The only significant interaction ef-

Table 1. Summary statistical results from a one-way ANOVA showing the effects of developmental stage on BWC and proportional BWC in *S. frugiperda*. SS = sums of squares, DF=degrees of freedom, BWC=body water content.

Trait	Effect	SS	DF	MS	F	*P*
BWC	Intercept	1.99	1	1.99	3115.48	< 0.001
	Developmental stage	1.25	7	0.18	278.72	< 0.001
Proportional BWC	Intercept	99.48	1	99.48	51920.61	< 0.001
	Developmental stage	0.85	7	0.12	63.45	< 0.001
	Error	0.29	152	0.00192		

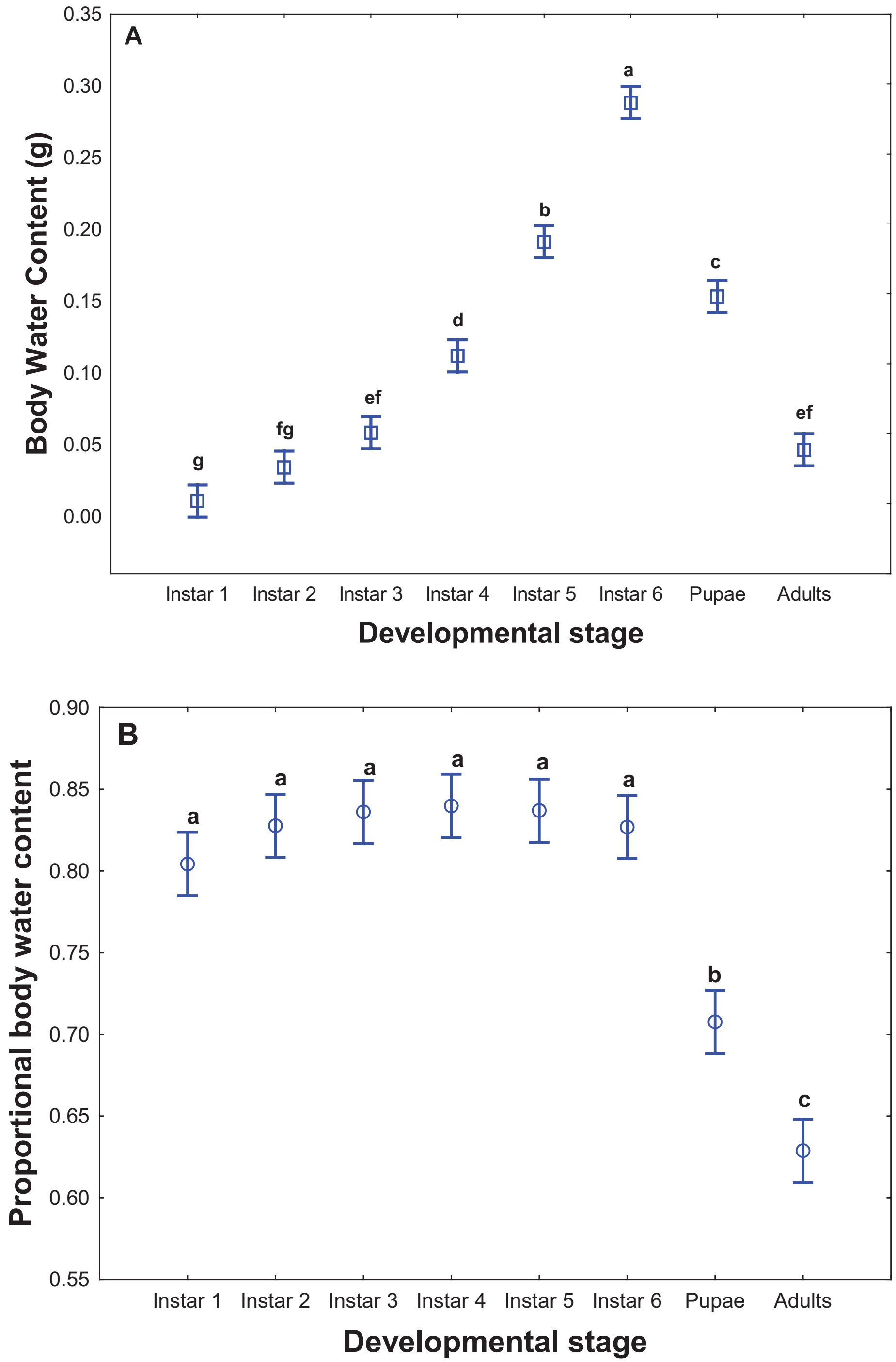

Figure 1. *Spodoptera frugiperda*'s body water content **A** and the proportional body water content **B** for larvae (all six instars), pupae and adults (48 h old). Error bars represent 95% confidence limits (N = 20). Means with the same letter are not significantly different from each other.

Table 2. Summary statistical results from factorial analysis using generalized linear model (GLM) on the effects of desiccation, developmental stage and interaction thereof on *S. frugiperda* water loss rates. All analyses were done in R version 3.3.0. DF = degrees of freedom.

Effect	DF	χ^2	*P*
Desiccation	1	0.05	0.65
Developmental stage	7	765.66	<0.001
Time	3	10.51	<0.001
Desiccation *Developmental stage	7	52.19	<0.001
Desiccation *Time	3	0.54	0.546
Developmental stage*Time	21	8.83	0.033
Desiccation *Developmental stage*Time	21	2.42	0.985

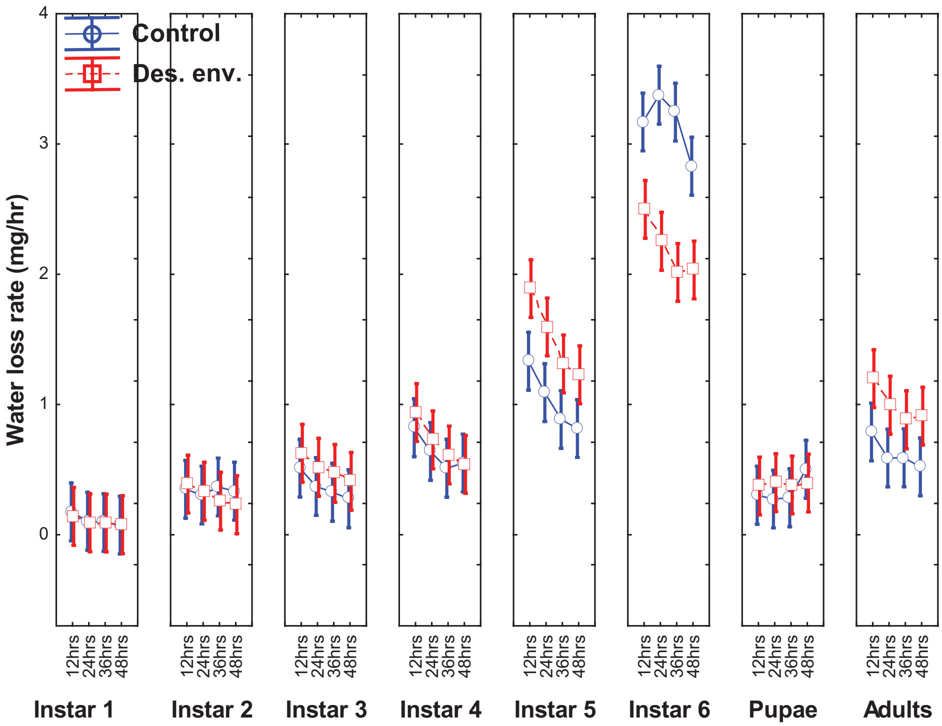

Figure 2. Water loss rates of *S. frugiperda* larvae (instars 1–6), pupae and adults following 12, 24, 36 and 48 h desiccation exposure at 7% RH. Error bars represent 95% confidence limits (N = 20 per treatment group). Des. env. = desiccation treatment (7% RH).

fect was found between treatment and developmental stage (Table 2). No mortalities were recorded for all individuals in each developmental stage during the WLR experiments.

Effects of desiccation pre-treatment on CTLs and fecundity

Following desiccation pre-treatment, all individuals and across all developmental stages survived acclimation stress before subsequent CTLs and fecundity experiments. Desic-

Table 3. Summary statistical results from a factorial ANOVA (CT_{min} and CT_{max}) and one-way ANOVA (fecundity) showing effects of desiccation pre-treatment, developmental stage and interaction thereof on *S. frugiperda* critical thermal limits (CT_{min}, CT_{max}) (larvae and adults) and adult fecundity, measured as number of 1st instar neonates larvae produced during a 7 days incubation period. SS = sums of squares, DF = degrees of freedom, CT_{min} = critical thermal minimum, CT_{max} = critical thermal maximum.

Trait Effect	SS	DF	MS	F	*P*
CT_{min} Intercept	6796.501	1	6796.501	27892.11	<0.001
Developmental stage	455.48	6	75.914	311.54	<0.001
Desiccation pre-treatment	0.350	1	0.350	1.44	0.232
Developmental stage* desiccation pre-treatment	23.001	6	3.834	15.73	<0.001
Error	64.817	266	0.244		
CT_{max} Intercept	655463.3	1	655463.3	58937.37	<0.001
Developmental stage	290.5	6	48.4	4.35	<0.001
Desiccation pre-treatment	47.7	1	47.7	4.29	0.039265
Developmental stage * Desiccation pre-treatment	155.1	6	25.8	2.32	0.033290
Error	2958.3	266	11.1		
Fecundity Intercept	5995405	1	5995405	852.7159	<0.001
Desiccation pre-treatment	3497	1	3497	0.4974	0.485
Error	267176	38	7031		

cation pre-treatment effects were generally dramatic for CT_{min}, CT_{max} and across tested developmental stages ($P > 0.01$) (Table 3). For example, desiccation pre-treatment impaired cold tolerance (higher CT_{min} temperature) in 1st instar larvae while improving the same trait (lower CT_{min} temperature) in 2nd and 3rd instar larvae (Fig. 3A). Similarly, desiccation pre-treatment impaired heat tolerance (lower CT_{max} temperature) in 5th larval instars while it appeared to have no significant effect on all other treated developmental stages (Fig. 3B). Both controls and desiccation pre-treated adults recorded significantly lowest CT_{min} (highest cold tolerance) while the rest of the treated developmental stages appeared to have CT_{min} ranging between 4.7 and 6.5 °C (see Fig. 3A). A significant interaction effect in CT_{min} was recorded between developmental stage and desiccation pre-treatment ($P < 0.001$) (Table 3). Similar to CT_{min}, the interaction effects of developmental stage × desiccation pre-treatment were significant for CT_{max} ($P < 0.05$) (Table 3).

Desiccation pre-treatment did not significantly influence fecundity ($P > 0.05$). The number of 1st instar neonates recorded in controls versus desiccation pre-treatments was 377.8 ± 10.43 and 396.5 ± 6.87 respectively (Table 3, Fig. 3C).

Discussion

One of the key environmental stresses insects face in both natural and agroecosystems is dehydration (Gibbs et al. 1997). Given that the incidences and severity of droughts and high temperature stress will increase with climate change in tropical regions, the population persistence of insects may be hinged on adaptation to these two stresses, either through high basal tolerance or integrated stress resistance (Guedes et al. 2015; Bujan et al. 2016). While Botswana is arid to semi-arid, microclimatic conditions experienced

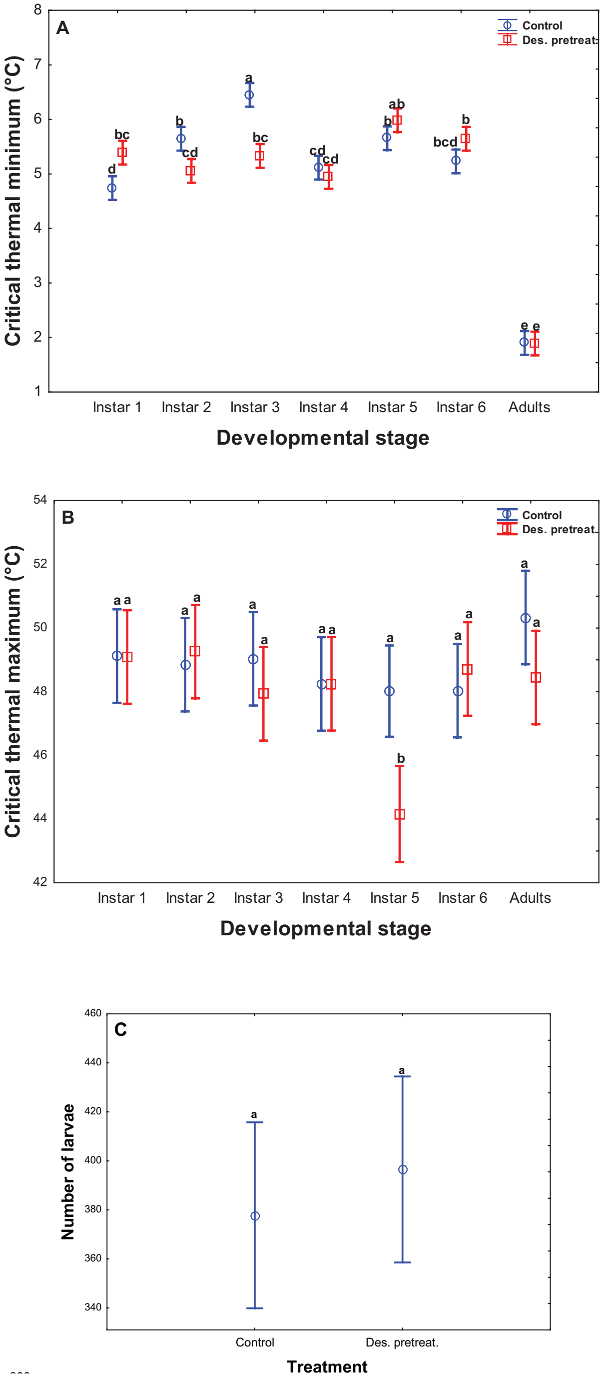

Figure 3. Effects of desiccation pre-treatment (des. pretreat.) on critical thermal minimum **A** critical thermal maximum **B** for *S. frugiperda* larvae (all six instars) and adults and **C** fecundity of *S. frugiperda* adults. Error bars represent 95% confidence limits (N = 20) and means with the same letter are not significantly different from each other.

locally show that *S. frugiperda* life stages likely experience episodes of high temperatures and desiccation, associated with heat waves and droughts during recent austral summers (see Keosentse et al. 2021). This indicates frequent exposure to overlapping acute and chronic environmental stresses, such as heat and desiccation. Our results show differential levels of water balance traits (basal BWC, proportional BWC and WLRs following desiccation) across developmental stages. Furthermore, our results also show dramatic effects of desiccation pre-treatment on physiological traits (CTLs), but without apparent structuration while effects of desiccation pre-treatment on fecundity were not apparent.

Body water content increased with larval instar age, with 1st and 6th larval instars recording the lowest and highest BWC respectively. The result is in keeping with Tarusikirwa et al. (2021) who recently reported similar trend in a related lepidoptera, *Tuta absoluta*, with larval instars' BWC increasing with age. In addition, this result is also consistent with findings by Bujan et al. (2016) that showed BWC increases with age and body size, likely extending the significant role of body size on BWC. Furthermore, body water reserves may be limited in smaller relative to larger developmental stages owing to higher surface area to body mass ratio (Krupp et al. 2020). This characteristic also makes smaller developmental stages more vulnerable to water loss than larger ones. This result suggests that early larval neonates may be more vulnerable to desiccating environments than the later stages that have higher BWC and relatively lower surface area to volume ratio. Bujan et al. (2016) reported higher BWC in canopy ants (larger insects) than understory ants (smaller insects). A comparison of 4th–6th instar larvae and adults showed that late larval instars had higher BWC than adults in keeping with Tarusikirwa et al. (2021) who reported higher BWC in *T. absoluta* 4th instars than adults. This may be attributed to their diets and habitat environments. Larvae feed on plant parts, full of sap that may provide higher amounts of water. Furthermore, adult *S. frugiperda* may be more 'terrestrial' than larval stages; the latter are always within microhabitats of the host plants, where they are protected from desiccation. This may partly account for the lower BWC of adults relative to the late instar larvae. This finding is congruent with Yoder et al. (2003) who reported higher BWC in *Stethorus nigripes* larvae than adults. In addition, pupae had higher basal BWC than adults. Indeed, from an evolutionary perspective, most 'immobile' developmental stages (e.g. pupae) always compensate for stress resistance through exhibiting inherent basal higher stress resistance than more mobile (e.g. adults) stages that can behaviorally compensate (Chown and Nicolson 2004). This partly accounts for the higher basal BWC of the pupae relative to adults reported here. Benoit et al. (2007) nevertheless reported higher BWC in 3rd versus 4th larval instars of *Belgica antarctica* suggesting ambivalent effects on the effects of developmental stage and body mass on basal BWC. Nevertheless, adults recorded lower proportional BWC than larval instars, in contrast to Tarusikirwa et al. (2021) who reported higher proportional BWC in *T. absoluta* adults relative to larvae. This suggests that proportional BWC in insects may be species and developmental stage dependent. We speculate here that a lower proportional BWC for adults may mean that adult *S. frugiperda* may use other mechanisms for avoiding desiccation. For example, adults are nocturnal (are mostly active during humid evenings) (Rojas et al. 2004) and hide under host leaves during desiccating daytime environments (Rwomushana 2019). This may mean that behavioral adaptation may be more important for desiccation tolerance in adults relative to other developmental stages.

Water loss rates increased with age amongst larval instars, consistent with the increase in BWC with age across larvae. First and 6th larval instars recorded the lowest and highest WLRs respectively and across the recorded times (12–48 h). These results are closely linked with BWC amongst larval instars since it also increased with larval instar age. This result is contrary to Benoit et al. (2007) who reported higher WLRs in 3rd than 4th larval instars of *B. antarctica*. Dehydration resistant insects are usually associated with higher lipid content or a heavily waterproofed cuticle that reduces water loss (Benoit et al. 2007). The variation in WLRs reported here may be linked to differential body lipid content among larval instars. Furthermore, significant differences in WLRs were only evident for 5th and 6th instar larvae. This may be because of their basal higher BWC which may allow desiccation pre-treatment responses to be evident as compared to early instars, whose relatively lower BWC may result in desiccation pre-treatment having little effects on plasticity of WLR. High WLRs recorded in the controls in instar 6 could be a combination of dehydration and mass loss due to consumption of reserves for energy since insects were not fed during the experiment. Decreased WLRs in the 6th instar following desiccation pre-treatment may also be due to the stress induced sit and wait strategy (Lighton and Fielden 1995). When exposed to stress, insects may reduce their metabolic activity hence reducing energy consumption and lowering water loss through gaseous exchange due to decreased respiration (Lighton and Fielden 1995). However, desiccation pre-treatment led to an increase in WLR in the 5th instar larvae, albeit not significant. Nevertheless, a gradual decrease in WLRs over time in 4th, 5th and 6th instars as well as adults relative to other developmental stages indicate that water balance may be life stage or cuticular permeability dependent, hence suggesting the role of these developmental stages (4th, 5th, 6th instars and adults) in surviving high temperature and desiccating arid African environments. Differential WLRs (low overall magnitude) recorded here following exposure to desiccating environments may highlight enhanced water conservation under constant organismal activity levels. Furthermore, it may also highlight potential organismal reduced metabolic activity during periods of stress, reflecting onto lower energy consumption as well as lower water loss through gaseous exchanges' due to decreased respiration (Benoit et al. 2007). While cuticular permeability was not measured across life stages in the current study, this warrants further investigation to elucidate these differential responses amongst life stages. Although pupae recorded higher BWC than adults, interestingly their WLRs were lower than those of adults. This supports the notion that immobile stages have developed inherent survival mechanisms given their inability to behaviorally thermoregulate under stressful desiccating environments. Pupae have a hard shell/cocoon that prevents abrasion of surface water-proofing layer of the cuticle hence improving desiccation resistance (reviewed in Danks 2000).

Our results showed improved cold tolerance (lower CT_{min}) for 2nd and 3rd larval instars following desiccation pre-treatment indicating some cross tolerance effects.

This is in consonance with Mutamiswa et al. (2018), who reported an enhanced CT_{min} in related species, *C. partellus*, *B. fusca* and *S. calamistis* larvae following desiccation pre-treatment. In addition, our results are in keeping with Bayley et al. (2001) who reported improved cold tolerance following desiccation stress in soil collembolan, *Folsomia candida*. These plastic responses in 2^{nd} and 3^{rd} larval instars indicate fitness and survival edge relative to other larval instars under desiccating environments. Although adults did not show any plastic responses following desiccation pre-treatment, they recorded the lowest CT_{min} indicating highest cold tolerance amongst all tested developmental stages outside acclimation responses. While insects have been reported to upregulate heat shock proteins (Hsps) synthesis in response to desiccation stress (e.g. Feder and Hofmann 1999), Hsps also improve cold tolerance (Chown and Nicolson 2004). Thus, this potential overlap in resistance mechanisms, albeit not measured here, may account for this integrated stress resistance in our results. Furthermore, desiccation pre-treatment impaired cold tolerance (higher CT_{min}) than controls, while it had no effects on all other measured parameters. Impairment of cold tolerance following desiccation pre-treatment suggests a cost of desiccation on cold tolerance, which may be developmental stage dependent. These fitness costs may be caused by the cumulative effects of both the sub-lethal desiccation pre-treatment and the cold tolerance traits that negatively affect fitness traits. Coupled with no acclimation effects of all other treatments for CT_{max}, this may point to a cross-susceptibility to temperature (especially heat) and desiccation across *S. frugiperda* development. Similar trends have been reported in *Sarcophaga crassipalpis* (Tammarielo et al. 1999) as well as *S. calamistis* and *C. partellus* larvae (Mutamiswa et al. 2018). Given these differential CTLs following desiccation pre-treatment in the current study, this suggests that physiological stress responses are life stage and trait dependent in *S. frugiperda* and that plastic interactive effects of desiccation and temperature may not yield significant beneficial acclimation responses.

Desiccation pre-treatment did not significantly affect fecundity in *S. frugiperda*. This suggests that invasive *S. frugiperda* still remains competitively fecund under highly desiccating arid environments and this trait may highly contribute to its rapid establishment in dry and hot tropical environments. The results are in agreement with Tajeda et al. (2016) who reported a similar trend between desiccation acclimated and control *A. ludens*. However, our results are in contrast with Benoit et al. (2010) who reported significantly lower fecundity in aquatic *Culex pipiens* following multiple bouts of dehydration. Nevertheless, the lack of dehydration effects on fecundity reported here may be a key determinant for increasing propagules of *S. frugiperda* when introduced in novel, arid and stressful habitats. Our fecundity assays were done following desiccation pre-treatment of adults only following acute acclimation pre-treatment. Given that more chronic acclimation treatments and/or carryover effects cascading into next life stages may alter adult fitness, future studies should focus on pre-treating earlier developmental stages under these aforementioned conditions to elucidate their effects on reproductive capacity of this invasive insect pest.

Conclusion

This work reports developmental stage differences in water balance and performance of *S. frugiperda* and implications on potential invasion under changing environments. Our results show that 1) basal BWC increased with age among larval instars and that this trend was also consistent with WLRs. However, proportional BWC seemed to decrease with developmental stage, and was lowest in adults. We also show that 2) WLRs increased with age in larval instars, and that pupae had lower WLRs than adults. However, the effects of desiccation environment on WLRs were beneficial for 6th relative to 5th instar larvae, suggesting the role of developmental stage on beneficial acclimation responses. Third, desiccation seemed to generally have more dramatic effects across development, suggesting complexity associated with acclimation responses that may be intertwined across development. Last, the effect of prior dehydration stress had no effects on fecundity, likely aiding the reproduction and fitness of this species in accumulating propagules under stressful arid environments. Physiological responses reported here may partly account for the thriving invasive populations of *S. frugiperda* in arid and semi-arid African habitats. Future studies should focus on understanding physiological mechanisms underlying water conservation in this invasive species. Similarly, more work may be needed to refine and make more conclusive plastic interactive effects of temperature and desiccation on *S. frugiperda* fitness traits. This information provides insights into understanding invasive species adaptation under desiccating environments and is significant in predicting spatio-temporal invasive pest outbreaks under changing abiotic environments.

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Threats at home? Assessing the potential ecological impacts and risks of commonly traded pet fishes

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Abstract

Invasive alien species (IAS) are major drivers of global biodiversity loss, and the poorly regulated international pet trade is a source of emerging and future invaders. Predictions of the likely ecological impacts and risks of such IAS have been significantly enhanced in recent years with new metrics, which require application to many more actual and potential IAS. Hence, this study assesses the potential ecological impacts and risks of two readily available pet trade species: goldfish, *Carassius auratus*, a species with non-native populations worldwide; and white cloud mountain minnow, *Tanichthys albonubes*, a species with a limited invasion history to date. First, we compared the *per capita* feeding rates of these non-native species with two European trophically analogous natives – the stone loach, *Barbatula barbatula*, and the common minnow, *Phoxinus phoxinus* – using the Comparative Functional Response method. Second, we used foraging experiments in conspecific pairs to determine synergistic, neutral or antagonistic intraspecific interactions. Third, we performed novel object experiments using the two pet trade species to assess boldness, a known "dispersal enhancing trait". Goldfish had the highest maximum feeding rates of the four species, while white cloud mountain minnows had the lowest. Neutral interactions were observed for all four species in the paired foraging experiments, with goldfish having the highest consumption and white cloud mountain minnows having the lowest. Goldfish demonstrated greater boldness, being more active during the experimental trials and more likely to approach a novel object than white cloud mountain minnows. Further, combining maximum feeding rates, boldness and species availabilities from our survey of pet shops, we assessed the relative invasion risks (RIR) of the two non-natives. This highlighted goldfish as the higher risk and most worthy of management prioritisation, mirroring its more extensive invasion history. We propose that such metrics have potential to direct future IAS policy decisions and management towards the ever-increasing rates of biological invasions worldwide.

Keywords
Behaviour, functional response, invasive alien species, pet trade, propagule pressure, relative invasion risk

Introduction

The global spread of invasive alien species (IAS) is a major driver of biodiversity loss (IPBES 2019), and can lead to severe ecological, economic, social and health implications (Laverty et al. 2015; Cuthbert et al. 2021; Diagne et al. 2021). Increasing globalisation has facilitated new pathways for IAS to spread (Hulme 2009; Zieritz et al. 2016), with the number unlikely to saturate in the future (Seebens et al. 2018, 2021). One industry that has benefited from increasing globalisation has been the pet trade. Known to be poorly regulated (Raghavan et al. 2013), and with associated laws often poorly communicated and enforced (Patoka et al. 2018), it has facilitated the spread of a number of high-profile IAS around the world (Lockwood et al. 2019) and is deemed responsible for a third of all aquatic IAS (Padilla and Williams 2004).

While most species in the pet trade spend their entire lives in confinement, many are released or escape from producers, importers, retailers and owners, and can exert impacts on recipient ecosystems through predation, competition with natives, hybridisation, habitat degradation and the spread of disease and associated biota (Polo-Cavia et al. 2010; Kraus 2015; Mrugała et al. 2015; Emıroğlu et al. 2016; Patoka et al. 2016, 2020; Lozek et al. 2021). Reasons for release include displays of aggression, increasing size, and maintenance costs (Duggan et al. 2006; Fujisaki et al. 2010; García-Díaz et al. 2015), or high reproductive output leading to overstocked tanks (e.g. marbled crayfish, *Procambarus virginalis*: Chucholl et al. 2012). Furthermore, prayer release, the Buddhist and Taoist practice in which the liberation of animals is thought to improve karma, is an overlooked introduction pathway that often involves releasing animals acquired from pet stores (Liu et al. 2012; Magellan 2019; Wasserman et al. 2019; Marková et al. 2020). The likelihood of IAS establishment is heavily dependent on propagule pressure (the number, frequency and viability of individuals of each species released), and in the context of species in the pet trade, likelihood of escape or release depends on species availability (Duggan et al. 2006; Chucholl 2013).

Two commonly traded species are goldfish, *Carassius auratus*, and white cloud mountain minnows, *Tanichthys albonubes*. The former is deemed one of the world's worst invasive species (Beatty et al. 2017), and has established populations around the world (Lorenzoni et al. 2018) in both lotic and lentic habitats (Kim et al. 2014). Indigenous to East Asia, it is one of the world's oldest domesticated fishes (Balon 2004; Novák et al. 2020). Known to be tolerant of a wide range of abiotic conditions, goldfish have been implicated in the spread of parasites (Mouton et al. 2001), the decline of native fishes (Deacon et al. 1964) and heavy grazing on aquatic plants (Richardson et al. 1995). Their method of benthic foraging is known to increase the turbidity of their habitats which in turn can affect competition with other species (Richardson et al. 1995), exacerbate algal blooms (Morgan and Beatty 2007), make them less visible to predators, and even allow them to regulate water temperature (Richardson et al. 1995). White cloud mountain minnows are endemic to southern China, and despite widespread popularity in the ornamental fish trade, they have a limited native distribution. Due to large-scale urbanisation and overexploitation by ornamental fishkeepers, they have been listed as a Class II protected animal in China's State Key Protected Animal List (Chan and Chen 2009). To date, there are a limited number of non-native populations – Colombia, Madagascar and Australia – but despite this and their global availability, little is known about their ecological impacts, actual or potential (Corfield et al. 2008). Our choice of these study species was informed by a 2017 survey of temperate/cold water species across twenty pet shops in Northern Ireland. With abiotic conditions such as temperature currently acting as a barrier to certain species in the pet trade from establishing and reproducing (Kalous et al. 2015; Standfuss et al. 2016), we highlighted which of the species recorded are capable of surviving temperatures of 10 °C or below. Of these, goldfish and white cloud mountain minnows were the most readily available (19/20 stores and 16/20 stores respectively: Suppl. material 1: Table S1).

Predicting ecological impacts and risks of such species was until recently deemed near-impossible (see Dick et al. 2014), with heavy reliance on invasion history, which is of little value prior to a species invading. However, new metrics combining traits and behaviour of species with easily measureable population metrics have enhanced impact and risk prediction (Dick et al. 2017b; Cuthbert et al. 2019b; Dickey et al. 2020). For example, the comparative functional response method, i.e. how consumption varies with resource availability for IAS relative to trophically analogous natives, has been highlighted as an effective way of assessing and predicting the ecological impacts of established, emerging and potential future non-native species (Dick et al. 2014).

One potential limitation of the comparative functional response method in measuring *per capita* consumption from the behaviour of individuals in isolation is that this misses the crucial role of intraspecific interactions inherent in group foraging, something particularly critical for assessing shoaling fish species. There are three broad categories of intraspecific interactions: neutral, antagonistic (prey risk reducing: Livernois et al. 2019), and synergistic (prey risk enhancing: Livernois et al. 2019), and accounting for this in the context of pet species releases, when small numbers of species might be released together, could prove valuable. There have also been calls for greater focus on behaviour in the study of invasive species, and specifically how the behaviour of successful IAS compare to less successful invaders (Rehage and Sih 2004). A number of behavioural traits have been deemed 'dispersal enhancing' (Rehage et al. 2016; Gruber et al. 2018). For example, boldness, i.e. how individuals behave in potentially risky situations (Réale et al. 2007), has been suggested to be a determinant of whether individuals are likely to disperse or remain sedentary, or whether they are short or long-distance dispersers (Fraser et al. 2001). How an individual, or group (see Kareklas et al.

2018), interacts with novel objects or shelter are commonly used methods of scoring boldness-like behaviour (Johnsson and Näslund 2018; McGlade et al. 2022).

Here, we thus sought to forecast the potential ecological impacts of goldfish and white cloud mountain minnows using three experiments: a comparative functional response study, an intraspecific paired feeding study, and a single and group boldness study; and then by combining this information into an adapted version of the Relative Invasion Risk (RIR) metric (Dickey et al. 2018). This three-pronged measure based upon the RIP metric (Dick et al. 2017b; Dickey et al. 2020) incorporates propagule pressure to assess the risk of a species establishing and exerting impact. We therefore used recent and new advances in IAS prediction metrics to assess the potential ecological impacts and risks of two non-native ornamental fishes, and discussed the general utility of these methods across the global pet trade.

Methods

Collection and husbandry

Goldfish were purchased from Carrick Pet Shop, Carrickfergus, Northern Ireland, over four batches due to availability (see Table 1 for further details). White cloud mountain minnows were purchased from Grosvenor Tropicals, Lisburn, Northern Ireland across two batches. For native comparators in the present study, we used two trophically analogous species (i.e. similar trophic ecology to the respective non-natives) commonly found in European temperate freshwater habitats. Firstly, we used the stone loach (*Barbatula barbatula*) as an example of a non-shoaling, benthic forager, more directly comparable to goldfish. Secondly, we used the common minnow (*Phoxinus phoxinus*), a species with a strong shoaling tendency (Magurran and Pitcher 1983) as a native analogue for the white cloud mountain minnow. Stone loaches were caught from the Minnowburn River by electrofishing on 6 August and transported to Queen's University Belfast School of Biological Sciences in a large barrel of continually aerated source water. Minnows were caught by hand net from Six Mile Water River on the 14 June 2019, and like the stone loaches, transported to the lab in aerated source water. All fish were maintained in glass holding aquaria (39.5 × 25 × 27 cm) within a controlled temperature laboratory with a temperature of 13 °C (+/- 1 °C) and a 12:12 light schedule, with experimental trials taking place in the same laboratory. Fish were given at least five days of adaptation to lab conditions before trials began. Holding aquaria contained an air stone, a filter and stones, plastic pipes and artificial plants to provide habitat enrichment. All fish were maintained on a diet of defrosted *Artemia* spp. (Monkfield Nutrition) and fed *ad lib* in the morning and the evening daily. To minimise disturbance to the fish, half water changes (as opposed to full water changes which require the potentially stressful removal of the fish) were performed weekly. Animal care was in accordance with institutional guidelines.

Table 1. Standard length and collection information for the four study species.

Study species	Standard length, mean ± SE	Origin	Collection date
Goldfish	49.6 mm +/- 0.76	Carrick Pet Shop, Carrickfergus	Batch 1: 16 July 2019, n = 8; Batch 2: 27 July, n = 12; Batch 3: 8 August, n = 12; Batch 4: 30 August, n = 8
White cloud mountain minnow	24.9 mm +/- 0.34	Grosvenor Tropicals, Lisburn	Batch 1: 5 July 2019, n = 20; Batch 2: 24 July, n = 20
Stone loach	61.4 mm +/- 0.90	Minnowburn River (54°32'54.7"N, 5°57'09.4"W)	6 August 2019
Minnow	41.2 mm ± 0.05	Six Mile Water River 54°42'16.6"N, 6°12'14.9"W	14 June 2019

Functional response

Fish were starved for 24 hours before functional response experiments began. Live bloodworm prey (*Chironomus* spp.), which all species were observed to readily consume, was offered as an ecologically relevant species in densities of 2, 4, 8, 16 and 32 (*n* = 3 per prey density, per species). Trials took place in acrylic tanks (22 cm × 17 cm × 22 cm) filled with 2 L of dechlorinated tap water that had been oxygenated overnight, and covered in masking tape so as to prevent any external visual disturbance. Trials ran for two hours, after which time the remaining alive prey were counted. Due to the high consumption rates of goldfish, additional prey densities (64 and 120) were offered to find a density that eventually led to the consumption rate reaching an asymptote for this species.

Paired feeding

Fish were starved for 24 hours prior to trials commencing. Trials took place in masking taped 10-L plastic aquaria (31.5 cm × 16.6 cm × 18.6 cm) filled with 6 L of dechlorinated tap water. Fish densities of 1× and 2× were used, with a constant density of bloodworm prey offered. This prey density was to be 60 bloodworms across all species, but this was increased to 240 for goldfish after pilots revealed their higher prey consumption rates relative to the other study species. To prevent any confounds ensuing from greater oxygen consumption in paired versus individual treatments, each arena was aerated via a portable, battery powered pump. Trials ran for 2 hours, after which time the number of live prey was counted.

Novel object test for goldfish and white cloud mountain minnows

This experiment occurred in batches of four fish individuals (6 batches, *n* = 24 individuals per species). Fish were selected from the holding tank and added individually to one of four masking taped plastic arenas (31.5 cm × 16.6 cm × 18.6 cm) containing 4 L of dechlorinated tap water, with four equally sized zones marked on the base, and a metal mesh placed over the first zone (Zone 1) to offer shelter (see Suppl.

material 2: Fig. S1). To avoid any effects of differing hunger levels (e.g. Nakayama et al. 2012), fish were given 10 defrosted *Artemia* spp. during a 30-minute adaptation period in their arenas (see Suppl. material 3: Fig. S2 for experimental procedure timeline), with an air stone added and green plastic sheets placed over the top. A 5-minute buffering period began with the commencement of recording using a camera (CX Action Camera, ACTIVEON Inc., U.S.A.) held above the arena with a retort stand, and removal of the air stone and the cover. Fish were exposed to two treatments, a "trial" (in the presence of the object) and a "control" (in the absence of the object) treatment to compare behaviour in the presence of the object with behaviour in an empty arena. Both treatments were 10 minutes long with their order randomised and balanced, so that half were trial then control, and half were control then trial (see Suppl. material 3: Fig. S2). This was done to prevent temporal confounds, such as the effect of familiarity with the arena or the effect of oxygen level on behaviour. Trial treatments were initiated by the dropping of the novel object (a blue plastic air stone splitter attached to string) into Zone 4, with the arenas subsequently recorded for another 10 minutes. Control treatments, in the absence of the novel object, commenced upon the removal of the novel object (when the order was trial-control) or the ten minutes prior to the addition of the novel object (when the order was control-trial). The string attached to the novel object was used to add and retrieve it in a way that minimised disturbance to the fish, as well as to ensure the novel object was confined to Zone 4 throughout. Once complete, all four fish were weighed, measured, and recorded being added into a fifth arena one at a time in advance of the group trial commencing. Again, a five-minute period took place before another paired trial/control treatment (order of treatments consistent with previous experiment) using a different novel object of differing shape and colour to maintain novelty – a small, grey, plastic koala figure again attached to string to enable retrieval (Suppl. material 3: Fig. S2). Behavioural analysis was conducted from video footage using BORIS v7.4.14 (Friard and Gamba 2016). For both individual trials and controls, the time spent in each zone was recorded, as was the summed total number of occurrences in each zone, which was used as a proxy for fish activity. For individual trials, the latency to approach the novel object, the number of approaches and the number of trials in which an approach was made was recorded, with an approach defined as contact between the fish and the object. In group trials, the latency was recorded as the time taken for the first fish individual to approach the novel object.

Data analyses

Functional response

Functional Responses (FR) were modelled using the 'frair' package (Pritchard et al. 2017).

Logistic regression of the proportion of prey consumed as a function of prey density was used to infer functional response types (Juliano, 2001). Here, a significantly negative first-order term is indicative of a Type II FR, whilst a significantly positive first-order term, followed by a significantly negative second-order term, is considered a Type III FR. As prey were not replaced as they were consumed, Rogers' random predator equation was used to model FRs (Rogers, 1972):

$$N_e = N_0\,(1–\exp(a(N_e\,h–T))) \qquad (1)$$

where N_e is the number of prey eaten, N_0 is the initial density of prey, *a* is the attack constant, *h* is the handling time and *T* is the total experimental period. Maximum feeding rates (1/*h*) were calculated under each treatment group. The *Lambert W* function was used to solve the random-predator equation (Bolker 2008). Non-parametric bootstraps (*n* = 2000) were used to generate 95% confidence intervals around FR curves, which were assessed visually for overlap.

Paired feeding

Per capita consumption was analysed via linear modelling with respect to species and predator density. Non-significant terms were removed stepwise (Crawley 2013), and Tukey's HSD post-hoc testing was performed using the lsmeans package (Lenth 2016).

Novel object

The number of individuals that approached the novel object was compared between species using Pearson's Chi-squared test, with Yates' continuity correction. Generalised linear modelling (Quasipoisson family) was used to assess the effect of species on the numbers of approaches and the individual and group latencies to approach. The latency to approach for groups was measured as the time taken for the fastest fish in the group to approach, which was compared with the time taken by the fastest of the four fish making up the batch in the individual tests (i.e. individual/group a variable in the model). For all models, backward reductions of non-significant terms and interactions facilitated the most parsimonious fits (Crawley 2013). The number of occurrences in each zone (which unlike approach measures occurred under both trial and control experiment treatments) was analysed with respect to species and experiment treatment (i.e. trial or control) with a generalised linear mixed model (Poisson family), with arena number (see Suppl. material 2: Fig. S1) as a random factor. Due to overdispersion, observation level random effects were used (Harrison 2014). The effect of experimental treatment (i.e. trial or control) and species on the time spent in the sheltered Zone 1 (time spent in other zones not analysed) was assessed using a linear mixed model with arena number as a random factor. Generalised linear models showed no significant effect of trial order (trial-control versus control-trial) on all the recorded measures.

Relative invasion risk

Dickey et al. (2018) defined invasion risk as the product of maximum feeding rate (derived from Functional Response experiments), a relevant life history trait as a proxy for the numerical response (e.g. lifespan, fecundity or lifetime fecundity), and pet propagule pressure (the proportional availability of the species from the pet stores surveyed). Relevant life history traits are often unavailable for pet species or based entirely upon observations in captivity. The adapted versions of the Invasion Risk (IR) metric used here are thus based upon maximum feeding rates taken from the functional response experiments, likelihood of approaching the novel object taken from the behaviour experiment as a life history trait alternative, and propagule pressure taken from a survey of temperate, freshwater fishes across twenty Northern Irish pet shops between the 31 January and 1 March 2017 (Suppl. material 1: Table S1). Firstly, we put forward a measure of IR that accounts for behaviour (IR_b):

$$IR_b = FR \times Boldness \times PPP \tag{2}$$

This is most similar to the version of IR featuring in Dickey et al. (2018). However, that study compared four trophically similar turtles and assessed their risk of establishing relative to each other in an ecosystem lacking native analogues. Here, one could argue that the study species are more trophically similar to the native species used rather than each other, with goldfish and stone loach two benthic foragers, and white cloud mountain minnows a shoaling species similar to common minnows. For that reason, a measure of risk should account for their impacts relative to the most trophically analogous natives, rather than each other. We therefore introduce a second IR measure that accounts for behaviour and trophic level, IR_bT. This incorporates a measure of relative maximum feeding rate (FR_r) of each pet trade species (FR_inv), taking account of the feeding rates of the closest native trophic analogues (FR_nat):

$$FR_r = \frac{FR_{inv}}{FR_{nat}} \tag{3}$$

Using this measure can allow multiple pet trade species from different taxonomic groups, with different ecological roles, and hence different trophic analogues, to be visually compared and prioritised, provided the measure of boldness (or other trait) is relevant for all. We can therefore define our second measure of IR (IR_bT) as:

$$IR_{bT} = FR_r \times Boldness \times PPP \tag{4}$$

As in Dickey et al. (2018), these three-pronged risk assessment metrics can be displayed visually using three dimensional graphs, with invasion risk increasing towards the top right corner. Alternatively, the Relative Invasion Risk score can be calculated for both measures:

$$RIR_b = \frac{IR_{\mathrm{b.inv\ 1}}}{IR_{\mathrm{b.inv\ 2}}} \tag{5}$$

$$RIR_{bT} = \frac{IR_{bT \cdot inv1}}{IR_{bT \cdot inv2}} \tag{6}$$

Like the RIP score as proposed by Dick et al. (2017b), when the resulting RIR value is < 1, this predicts "invader 1" will be lower risk than "invader 2"; when RIP = 1, we can predict the risk of "invader 1" to be no different from that of "invader 2"; and when RIP is > 1, we can predict that "invader 1" will be higher risk than "invader 2".

All statistical analyses were carried out in R v.3.2.2 (R Core Development Team 2015). Scripts for functional response analysis and Relative Invasion Risk analysis available in the supplementary material of Pritchard et al. (2017) and Dickey et al. (2018) respectively.

Results

Functional Response

Prey survival in all control groups was 100%, and thus all prey mortality in experimental groups was attributed to fish predation, which was also directly observed. Type II functional responses were exhibited by all four species, as determined by significant negative first order terms (Table 2; Fig. 1). Goldfish had only the third highest attack rate but the shortest handling time, and hence a much higher maximum feeding rate than any of the other study species. White cloud mountain minnows exhibited the lowest attack rates and the longest handling times (and therefore the lowest maximum feeding rates, $1/h$, of the four study species). Stone loach had the second highest attack rate, and the second shortest handling time, and hence second highest maximum feeding rate after goldfish. Minnows had the highest attack rate, but the second longest handling time, after white cloud mountain minnows (Table 2; Fig. 1).

Paired feeding

Assessing the species given a fixed prey density of 60 (i.e. excl. goldfish), there was a significant effect of species on *per capita* consumption (linear model, LM: adjusted $R^2 = 0.68$, $F_{3,26} = 18.39$, $p < 0.001$; Fig. 2), with white cloud mountain minnows eating significantly less than stone loach ($p < 0.001$) and minnows ($p < 0.001$), and stone loach eating significantly more than minnows ($p = 0.002$). When goldfish (fixed prey density of 240) were included in the model (LM: adjusted $R^2 = 0.78$, $F_{4,35} = 31.85$, $p < 0.001$), their average *per capita* consumption rates were significantly higher than the others ($p < 0.001$ for all comparisons). Average *per capita* consumption was not significantly affected by predator density in either model (goldfish excluded: $p = 0.363$; goldfish included: $p = 0.302$).

Table 2. First order terms calculated from logistic regression to denote functional response type across all predator treatments. The significant negative first order term values across all four species indicate Type II functional responses for each predator. Attack rate (a), handling time (h) and maximum feeding rate (1/h) parameter estimates derived using Rogers' random predator equation (Eq. 1). '***' $p < 0.001$, '**' $p < 0.01$. '*' $p < 0.05$, '.' $p < 0.1$.

Species	Prey	First-order term	Attack rate (*a*)	Handling time (*h*)	Maximum feeding rate (1/*h*, prey per 2 hours)
Goldfish	Chironomid	-0.01**	2.26***	0.01***	217.64
White cloud mountain minnow	Chironomid	-0.09 ***	0.81.	0.35***	2.89
Stone loach	Chironomid	-0.06**	2.65***	0.02***	42.87
Common minnow	Chironomid	-0.11 ***	3.57**	0.09***	11.11

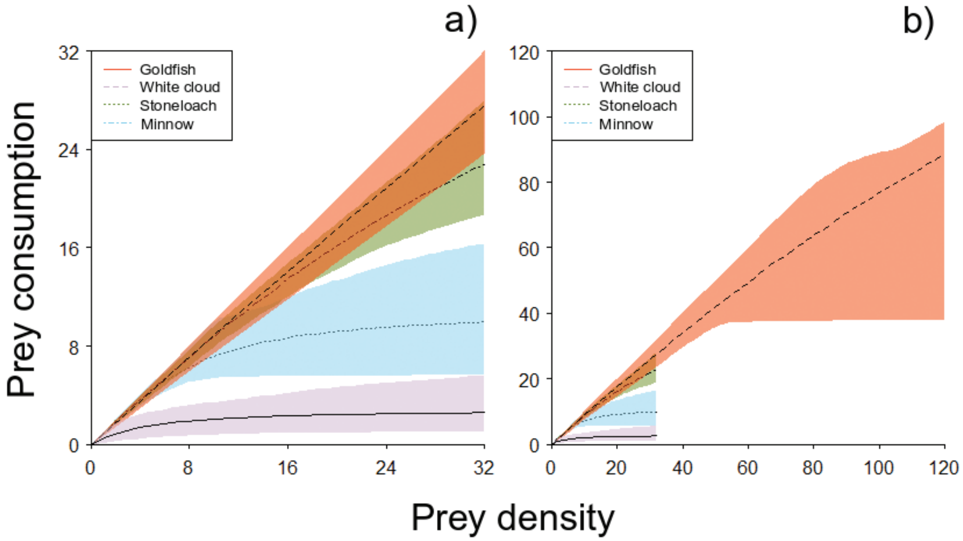

Figure 1. Functional response curves of goldfish, white cloud mountain minnow, stone loach and common minnow towards Chironomid prey. Shading represents 95% confidence intervals **a** all species compared over prey densities up to 32 **b** as per **a** but with prey dentities up to 120 to derive goldfish asymptote.

Novel object

Overall, goldfish were more likely to approach the novel object than white cloud mountain minnows (91.67% v 54.17% of trials out of 24 in which focal fish approached; $\chi^2 = 6.75$, df = 1, $p < 0.01$), however, of the individuals that did approach, the number of approaches did not differ by species. There was no significant effect of experimental order, i.e. trial-control versus control-trial, on the latency, number of approaches or activity (generalised linear models, GLM: $p = 0.255$, $p = 0.654$, $p = 0.795$). There were no significant effects of species or experiment type (i.e. single or group) on latency (GLM: $p = 0.571$, $p = 0.313$). Analysing activity levels, there were significant effects of species and

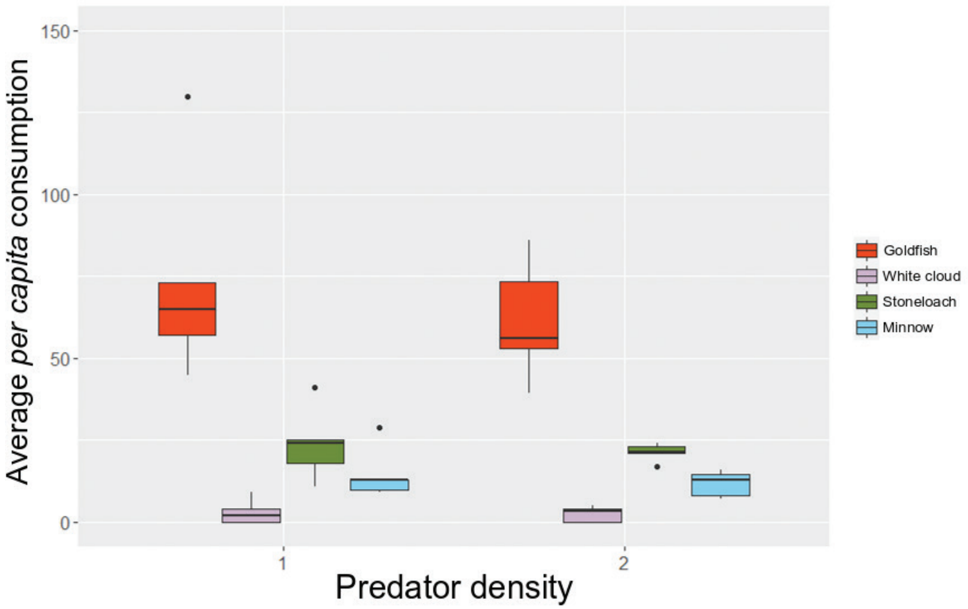

Figure 2. Average *per capita* consumption across single and pair predator densities in the group feeding trials. Fixed prey density of 60 bloodworms for white cloud mountain minnows, minnows and stone loach. Fixed prey density of 240 bloodworms for goldfish.

treatment type (i.e. trial v control), but no significant two-way interaction, with goldfish more active than white cloud mountain minnows ($z = 2.31$, $p = 0.02$: Fig. 3), and both species less active during trial experiments ($z = 2.03$, $p = 0.04$: Fig. 3). Assessing the effect of trial or control and species on the time spent in Zone 1 showed no significant finding.

Relative invasion risk

Using both RIR measures, goldfish were shown to have much higher invasion risks than white cloud mountain minnows, with both calculations giving scores > 1 (Table 3; Fig. 4). The RIR_b score was 151.56, with the difference in feeding rates the main driver of the large disparity (Table 3; Fig. 4a). Using RIR_{bT} however lessened this, giving a score of 39.29, due to a smaller relative maximum feeding rate of goldfish in relation to stone loach, the other benthic forager (Table 3; Fig 4b). Both scores highlight goldfish as a much greater invasion risk.

Discussion

In an increasingly globalised world, the need for methods to predict and prevent future IAS is vital (Diagne et al. 2021; Vilizzi et al. 2021). Here, we assessed two readily

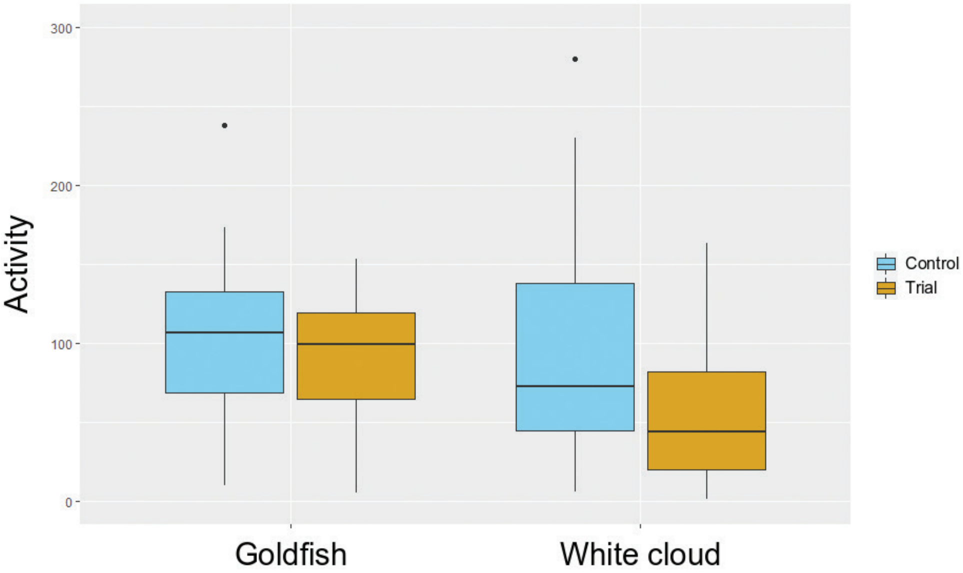

Figure 3. Goldfish and white cloud mountain minnow activity in control and experimental trials, as determined by the summed number of occurrences in each of the four zones.

Table 3. Relative Invasion Risk (RIR_b) and Trophic Relative Invasion Risk (RIR_{bT}) calculations, whereby RIR_b = maximum feeding rate (FR) × boldness (B) × pet propagule pressure (PPP), and RIR_{bT} = maximum feeding rate relative to trophically analogous native (FR_T) × boldness (B) × pet propagule pressure (PPP). The FR_{native} comparator for goldfish was the stone loach, and the native comparator for white cloud mountain minnow was the common minnow. The novel object approaches figure is the proportion of trials in which the species approached the novel object out of 24 trials. The Pet Propagule Pressure figure is the proportional availability of the species out of 20 surveyed pet shops (see Suppl. material 1: Table S1). IR_b is calculated as the product of FR, B and PPP. RIR_b is calculated by dividing the IR_b of goldfish by the IR_b of white cloud mountain minnow. IR_{bt} is calculated as the product of FR_T, B and PPP. RIR_{bT} is calculated by dividing the IR_{bT} of goldfish by that of the white cloud mountain minnow.

Species	FR	FR_{native}	FR_T	B	PPP	IR_b	RIR_b	IR_{bT}	RIR_{bT}
Goldfish	217.64	42.87	5.08	0.92	0.95	189.60	151.56	4.42	39.29
White cloud	2.89	11.11	0.26	0.54	0.80	1.25		0.11	

available species from the pet trade that are likely to be capable of surviving in a temperate zone: goldfish and white cloud mountain minnows. First, the comparative functional response (CFR) method compared these pet trade IAS with the trophically analogous native stone loach and common minnow. This method has proven robust in highlighting damaging invaders relative to trophically analogous native species or less damaging IAS based on their resource uptake rates (Bovy et al. 2014; Dick et al. 2014; Dodd et al. 2014). Second, we assessed the role that intraspecific group size plays on *per capita* foraging rates. Specifically, this was to reveal whether the presence of a conspecific led to intraspecific interactions that are neutral, i.e. average *per capita* feeding rates remained constant regardless of group size; synergistic, i.e. average *per capita* feed-

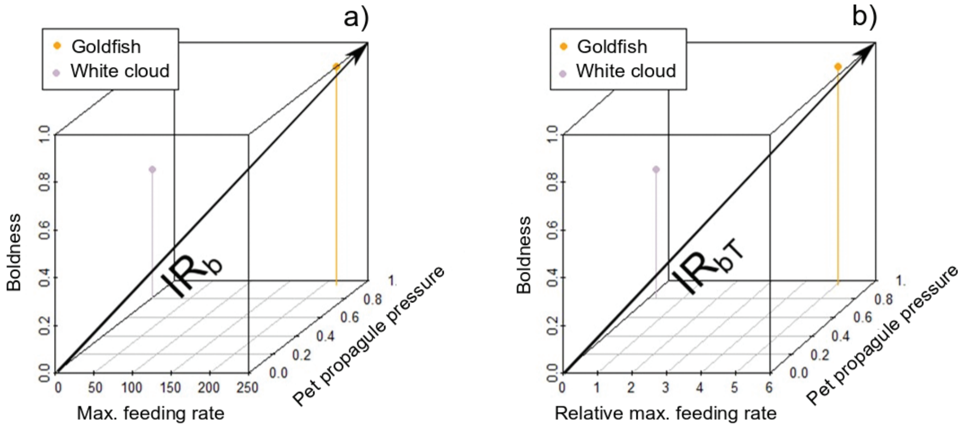

Figure 4. Three dimensional graphs showing Relative Invasion Risk (RIR) of goldfish and white cloud mountain minnows. RIR_b calculated as a product of maximum feeding rate, boldness, and Pet Propagule Pressure (PPP) **a** and RIR_{bT} calculated as the product of IAS maximum feeding rate divided by native analogue maximum feeding rate, boldness and PPP **b**. Invasion Risk increases from bottom left to top right of each plot.

ing rates increased in groups; or antagonistic i.e. group feeding reduced average *per capita* feeding rates (Livernois et al. 2019). Third, we compared goldfish and white cloud mountain minnow behaviour with and without the presence of a novel object in order to assess measures of boldness, a known "dispersal enhancing trait" (Gruber et al. 2018). Finally, this information was combined alongside availability measures from our pet store survey within two adapted versions of the Relative Invasion Risk (RIR) metric.

Functional response

All four study species exhibited potentially "destabilising" type II functional responses, however, there were clear differences in terms of attack rates, normally indicative of predation at low prey densities (Dick et al. 2014), and handling times and maximum feeding rates, used as an indicator of ecological impact (Dick et al. 2017a). Goldfish had only the third highest attack rate but the highest maximum feeding rate, indicative of a large ecological impact. With the study goldfish used having no known previous exposure to live prey, this suggests an adaptability to novel prey items, which has been demonstrated in prior experiments (Monello and Wight 2001). Despite being known to eat insect larvae in the wild throughout the year alongside zooplankton and detritus (Yi et al. 2004; Liang et al. 2008), white cloud mountain minnows had the lowest attack rates, and the lowest maximum feeding rates, suggesting a low ecological impact. Stone loach had the second highest attack rate and the second highest maximum feeding rate, while common minnows demonstrated the highest attack rate, but only the third highest maximum feeding rate. Chironomid larvae is known to feature in both native species' diets (Smyly 1955; Museth et al. 2010; Vinyoles et al. 2010), making similar percentage contributions (Frost 1943; Smyly 1955), and the differences here can likely be ascribed to method of predation. Stone loach, with eyes located dorsally, tend not to rely on sight when foraging, instead using their barbels to sense prey (Smyly 1955), which may in turn have led to the minnow offering a greater predatory threat at low prey densities.

Paired feeding

This was done to address a shortcoming of recent impact assessment metrics (Dickey et al. 2020) which define impact as the product of the maximum feeding rate, as derived from functional response experiments, with a proxy of numerical response, normally species abundance or density. Such metrics have an inherent assumption that feeding rate increases linearly with predator numbers (i.e. neutral intrapecific interactions), and in turn fail to account for possible synergies or antagonisms between predators. The type of interaction may offer valuable insights into establishment and spread at different stages of the invasion process. For example, synergistic interactions, i.e. a lack of aggression towards conspecifics, have been thought to facilitate the success of the Argentine ant (*Linepithema humile*) in its introduced range (Suarez et al. 1999). Conspecific group foraging also confers numerous advantages in terms of finding and consuming food, and antipredator defence (Pitcher et al. 1982; Creel and Creel 1995), which may be of added importance in novel ecosystems.

Here, using our four study species across two different predator densities, a significant effect of species was found, but not for predator group size. In other words, we saw similar average individual feeding rates at both single and paired densities, and this was the case for all four species. The same pattern from the functional response experiment was shown, with goldfish having the highest consumption rates both singly and in pairs, with white cloud mountain minnows again having the lowest consumption rates. From this, we could therefore assign “neutral” rather than synergistic or agonistic interactions to all four species: a classification that here matches the inherent assumption of linearity of RIP. Neutral interactions for goldfish and stone loach were as expected as neither species is a shoaling species, with the former only truly social when breeding (Dunlop et al. 2006), and neither is known to be particularly aggressive towards conspecifics (unlike, for example, the benthic round goby, *Neogobius melanostomus*, Groen et al. 2012), with stone loaches typically found in small groups (Smyly 1955). However, the lack of effect of group size on foraging by the shoaling white cloud mountain minnows and common minnows was unexpected. It may suggest that the release of small numbers of white cloud mountain minnows would have neutral, and predictable additive effects. A study into the welfare implications of group size on white cloud mountain minnows (Saxby et al. 2010) showed no difference in the amount of “darting behaviour” – associated with predator avoidance, being chased or being unsettled – exhibited by groups of two fish relative to groups of 5 or 10. Similarly, there was no difference in time spent in an area of area of environmental enrichment relative to groups of 10, and fewer aggressive acts per individual than in groups of 5 and not significantly different to the number in groups of 10. However, groups of two still took significantly longer to start feeding relative to groups of 5 or 10, though significantly less than for individual fish, indicating decreased welfare from an unnaturally small group size. Fish in small shoals are known to spend less time foraging than fish in larger shoals (Magurran and Pitcher 1983), and we therefore encourage future studies to investigate the effects of higher density treatments, especially for comparing the white cloud mountain minnow and common minnow.

Novel object

While invasion success depends on myriad factors and species traits, behaviour has been shown to play a major role (Weis 2010; Chapple et al. 2012), with a number of behavioural traits associated with invasion success and dispersal, such as boldness. One frequently used method to assess where individuals lie along the bold-shy axis and their degree of neophilia has been the novel object test, whereby satiated individuals are presented with an unfamiliar object, and the number of interactions are recorded per unit time. In novel object tests the approach is elicited by the object and not by any associated reward, as opposed to tests featuring novel food items for example (Kareklas et al. 2018), and therefore is a measure of attraction to intrinsically rewarding novelty (Griffin et al. 2016). This motivation to approach novel stimuli is deemed critical to persisting in new environments in which they lack specific knowledge for survival (Griffin et al. 2016). Here, we assessed boldness in the form of the likelihood of approaching the novel object, the latency to approach the novel object (both individually and in conspecific groups), the number of approaches, activity and the residency in the sheltered Zone 1 in the experimental arena. While a number of studies to date have compared measures of dispersal enhancing traits between non-native and native species, or invasion frontier versus long-established non-native populations (Rehage et al. 2016), we compared the two pet trade species: goldfish as a species with an extensive invasion history; and white cloud mountain minnows as one with a limited invasion history. In doing so, we found that goldfish were more likely to approach the novel object than white cloud mountain minnows, and were more active. Despite known tradeoffs associated with boldness (Stamps 2007), high boldness and activity levels have been shown to enhance feeding opportunities (Brownscombe and Fox 2013), survival in the presence of predators (Smith and Blumstein 2010; Blake et al. 2018), and boldness has also been shown to correlate to dispersal in the field (Fraser et al. 2001), as well as measures of reproductive success in other taxa (e.g. Collins et al. 2020). There was also a significant effect of trial/control on general activity levels, which may indicate a level of behavioural flexibility for both species, and context-specific adjustments of behaviour have been shown to be beneficial for a number of successful IAS (Sol et al. 2002; Cure et al. 2014). Of course, the degree of boldness demonstrated during the novel object test will have been determined by perception of costs by individuals of both species. While the specific drivers of the behaviour of both species are beyond the scope of this study, goldfish are larger than the white cloud mountain minnows, and greater size has been shown to facilitate greater risk taking, due to lower likelihood of predation (Ioannou et al. 2008). However, goldfish also have to balance this against colouration that makes them more visible to potential visual predators (their ability to change the turbidity of water bodies is deemed a survival benefit for this reason:

Richardson et al. 1995). It is important to note that black goldfish colour morphs exist and, conversely, gold colour morphs of *T. albonubes*. The degree to which colour affects behaviour and establishment success is a worthy avenue for further study. The mechanisms behind the costs and benefits perceived by individuals of both species are also shaped by evolutionary history in their native range (e.g. the nature of the predators of both species when in the wild), the number of generations of domestication (i.e. goldfish have been selectively bred for over 1000 years: Chen et al. 2020) and physiological drivers. Ultimately, these combine to give goldfish higher exploratory tendencies and, when combined with higher feeding rates, demonstrate what Rehage et al. (2016) describe as an 'invaders syndrome', which corroborates with them having established worldwide, being known to be highly motile in novel ecosystems (found to move up to 4 km.day^{-1}: Kim et al. 2014), and being expected to further expand their range in the near future (Beatty et al. 2017). Also, as a species regularly kept in outdoor ponds, they are at heightened risk of further spread (Copp et al. 2010; Patoka et al. 2017).

Relative Invasion Risk, implications and future studies

Our two measures of RIR allowed the key findings from the functional response and behaviour experiments to be combined alongside a measure of propagule pressure based on availability in the pet trade, to give an overall measure of invasion risk. Due to the lack of information available for our study species in the wild, we used a measure of boldness rather than life history traits (as used in Dickey et al. 2018), or alternative proxies of numerical response (Dickey et al. 2020). However, connections between boldness and reproductive fitness have been demonstrated. For example, bolder male zebrafish (*Danio rerio*) were shown to fertilise more eggs (Ariyomo and Watt 2012), boldness was associated with sperm number in male guppies, *Poecilia reticulata* (Gasparini et al. 2019), and moving beyond fish species, boldness and reproductive success are linked for black-legged kittiwakes, *Rissa tridactyla* (Collins et al. 2020). The two measures of RIR used in this study both demonstrated the greater risk posed by goldfish, albeit in subtly different ways. While RIR_b is closer in nature to the measure of RIR previously proposed (Dickey et al. 2018, 2020), it offers a risk assessment measure only focused on comparing the risks of the pet trade study species, that similarly allowed direct comparison between four pet trade turtles introduced into northern Europe with a lack of native analogues in Dickey et al. (2018). That is not the case here, and while a method of comparing two cyprinid fish species is valuable, their respective roles in a recipient ecosystem could prove very different. For that reason, a measure of RIR (such as our RIR_{bT}) that accounts for their feeding rates relative to trophically analogous native species, provides policy makers with a method that allows multiple pet trade species, across multiple taxonomic groups (e.g. fish, crayfish, shrimps), and at multiple trophic levels, to be compared and prioritised for risk, provided there are native analogues. Indeed, policymakers could decide upon a threshold IR_{bT} number, beyond which imports or sales of that species could be curtailed, dovetailing with or, for countries outside of the EU, offering an alternative to the list of Invasive Alien Species of Union Concern (Regulation 1143/2014).

It is important to note that functional responses, intraspecific interactions and behaviour of invasive species are not fixed, and they often change over the course of an invasion as the population is subjected to different selection pressures. This has been highlighted by a number of studies that have compared populations at the invasion frontier with long-established populations (Groen et al. 2012; Iacarella et al. 2015; Tarkan et al. 2021), populations in invaded ranges with those in the native range (Suarez et al. 1999; Howard et al. 2018), and in the context of pet species, those in captivity versus those that have established wild populations (Linzmaier et al. 2018). However, we propose that the methods used in this study stand to highlight goldfish as a species exhibiting traits that might "pre-adapt" it to establish, spread and exert ecological impact. As well as demonstrating a voracious appetite in this study, the species is known to have a highly adaptable, generalist diet, which can lead to extensive grazing on aquatic vegetation, or preying on amphibian eggs or aquatic invertebrates (Richardson et al. 1995; Monello and Wight 2001). They are also known to be highly tolerant of anoxic conditions (Fagernes et al. 2017), saline conditions up to 6 ppt (Luz et al. 2008), temperature (Ferreira et al. 2014) and their large maximum size combined with potentially long lifespan (Froese and Pauly, 2022), may make them more likely to be released by pet owners (Duggan et al. 2006).

Going forward, we encourage further impact assessment methods that account for propagule pressure, predatory impact and dispersal-enhancing behavioural traits, and propose that the RIR methods introduced here offer a way of doing so by combining such findings. While the study species here were selected based on availability from one pet shop survey, the global pet market is taxonomically dynamic, with major shifts in species availability over time (Lockwood et al. 2019), and therefore warrants continued observation. Alternative sources of information such as listings of the species being imported into or exported from a country, e.g. the US Law Enforcement Management Information System (LEMIS) database (Fujisaki et al. 2010; García-Díaz et al. 2015), and informal online marketplaces (Olden et al. 2021) are similarly in need of monitoring. The creation of a database whereby IR scores can be generated in real time as availability changes, could provide policy makers with a valuable information source and a means to reduce the risk proactively, instead of reactively (Simberloff 2006). It could also inform law changes and help make previous "dead letters" implementable (Patoka et al. 2018).

In terms of the constituent elements of RIR, comparative functional responses using alternative native prey species might offer further insights into foraging interactions, and for "benthic grazers" like goldfish, the addition of relevant substrate and different degrees of habitat complexity (Wasserman et al. 2016; Cuthbert et al. 2019a) may add greater realism. We advocate for future assessment measures that account for dispersal enhancing traits to do so alongside different biotic and abiotic contexts relevant across different invasion stages (Smith and Blumstein 2010), and niche similarity with any potential recipient system (Liu et al. 2020; Broennimann et al. 2021) and measures of survival and reproduction (Kouba et al. 2021), could aid the prioritising of potential IAS for study. Further, behaviour studies that assess the degree of naiveté towards native predators, e.g. through chemical or visual predator cues (Lönnstedt et al. 2012; McCoy et al. 2012; Martin 2014), could help determine the degree of "pre-adaptation" of potential pet shop IAS to novel recipient ecosystems, and therefore aid management prioritisation and regulation.

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Supplementary material 1

Table S1

Authors: James W. E. Dickey, Gareth Arnott, Ciara L. O. McGlade, Andrew Moore, Gillian E. Riddell, Jaimie T. A. Dick

Data type: Csv file.

Explanation note: Temperate, freshwater species recorded during Northern Ireland pet shop survey between the 31 January and the 1 March 2017. Numbers in the Availability column refer to number of pet shops out of the twenty shops surveyed that had the listed species present. Numbers in square brackets refer to shops were the species was not observed, but there was signage to indicate the recent presence of the species. Known invasion history determined from fishbase.de, with Y indicative of at least one established non-native population.

Link: https://doi.org/10.3897/neobiota.73.80542.suppl1

Supplementary material 2

Figure S1

Authors: James W. E. Dickey, Gareth Arnott, Ciara L. O. McGlade, Andrew Moore, Gillian E. Riddell, Jaimie T. A. Dick

Data type: Csv file.

Explanation note: Experimental set-up relating to the novel object experiment. Zone 1 covered with a metallic mesh to provide shelter for each fish, while the novel object was placed in Zone 4.

Link: https://doi.org/10.3897/neobiota.73.80542.suppl2

Supplementary material 3

Figure S2

Authors: James W. E. Dickey, Gareth Arnott, Ciara L. O. McGlade, Andrew Moore, Gillian E. Riddell, Jaimie T. A. Dick

Data type: Csv file.

Explanation note: Novel object experimental procedure.

Link: https://doi.org/10.3897/neobiota.73.80542.suppl3

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RESEARCH ARTICLE

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Advancing research on alien species and biological invasions

Genetic and morphological evidence indicates the persistence of Japanese mitten crab mitochondrial DNA in Europe for over 20 years and its introgression into Chinese mitten crabs

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Abstract

Cryptic biological invasions are largely unrecognised, leading to an underestimation of the number of invading taxa and their potential impacts. The Chinese mitten crab, *Eriocheir sinensis*, is a highly invasive species with serious economic and ecological impacts in Europe. Recently, mitochondrial DNA (mtDNA) of the Japanese mitten crab, *E. japonica*, has been discovered in populations from The Netherlands, Poland and Germany, but the taxonomic status and time of introduction of specimens carrying this mtDNA are uncertain. To this end, we investigated the morphology and variation of the mitochondrial cytochrome c oxidase subunit I (COI) gene of mitten crabs collected in central-western Europe between 1998 and 2020. Mitten crabs from Belgium harboured a Japanese mitten crab COI haplotype in 33% to 65% of individuals, even in our earliest samples from 1998. All other studied populations carried only Chinese mitten crab COI haplotypes. Morphologically, many of the juvenile Belgian mitten crabs showed intermediate traits between the two species, while all investigated adult mitten crabs, regardless of their mitochondrial haplotype or country of origin, were morphologically assigned to *E. sinensis*. This intermediate morphology of the juveniles and genetic-morphological discrepancy of adults suggests that Japanese mitten crabs introgressed with Chinese mitten crabs, which could have happened both before and after the introduction of mitten crabs to Europe. A specific Chinese mitten crab COI haplotype, found in Belgium, was previously only known from Vladivostok (Russia), where Chinese and Japanese mitten crab hybrids naturally occur. This Far East region is, therefore, a plausible source for at least part of the mitten crab mitochondrial diversity in Belgium.

Keywords
Belgium, introgression, mitochondrial DNA, mitten crab, morphometrics, museum collections

Introduction

Global expansion in travel and trade has greatly increased the number of invasive species (Everett 2000). However, both the numbers of invaders and the impacts of these species may be underestimated as a result of cryptic invasions (Carlton 1994). Cryptic invasions and potential admixture between species may be occurring in one of the "world's 100 worst invasive species", the Chinese mitten crab, *Eriocheir sinensis* H. Milne Edwards, 1853 (Lowe et al. 2000). Over 100 years ago, it arrived in Europe via ballast water of large shipping vessels and has become an unprecedented nuisance with staggering ecological and economic effects (Peters and Panning 1933; Clark et al. 1998; Rudnick et al. 2000; Herborg et al. 2003, 2005; Dittel and Epifanio 2009). Mitten crabs (*Eriocheir* spp.) are catadromous species, reproducing in seawater, but spending most of their life cycle in freshwater (Peters and Panning1933; Clark et al. 2006; Naser et al. 2012; Schoelynck et al. 2021). The Chinese mitten crab is indigenous to East Asia, where it is distributed along the coast of the East China Sea, Yellow Sea and Bohai Gulf up to Vladivostok in Russia (Wang et al. 2008; Sui et al. 2009; Xu et al. 2009; Kang et al. 2018).

In a global assessment of mitten crab diversity, Hayer et al. (2019) synthesised all available sequences of the mitochondrial gene for cytochrome oxidase c subunit I (COI hereafter) for mitten crabs from GenBank and the Barcoding of Life Data System (BOLD, https://www.boldsystems.org/). They found that some specimens of presumed Chinese mitten crabs collected in The Netherlands, Poland and Germany between 2009 and 2015 yielded COI sequences of the Japanese mitten crab, *E. japonica* (De Haan, 1835). The Japanese mitten crab is indigenous to the main Japanese islands, eastern and southern parts of the Korean peninsula and Vladivostok in Russia (Xu et al. 2009) and had previously not been known to establish non-native populations. We set out to investigate the extent of *E. japonica* genetic diversity throughout Europe since 1998 and to evaluate the potential evidence for hybridisation between Chinese and Japanese mitten crabs in Europe using morphological comparisons.

Methods

A total of 141 mitten crabs were collected for morphological and genetic assessment by local fishermen or hand-caught between 1998 and 2020: 65 crabs from the Rivers Eider, Elbe, Weser and the Kiel Canal in northern Germany, 20 crabs from the Vistula Lagoon in Poland and 56 crabs from Oostende (North Sea) and the Schelde River Basin in Belgium (Table 1). They were frozen or immediately preserved in 75% to 99% ethanol. Specimens collected in Belgium between 1998 and 2005 are deposited in the National Taiwan Ocean University (NTOU). Tissue vouchers of all remaining specimens are preserved in the Zoological Museum of Kiel University, where also a subset of whole specimens from Germany, Belgium and Poland, collected between 2019 and 2020, is stored.

We extracted genomic DNA from about 1 mm^3 of pereopod muscle tissue with the Chelex method (Walsh et al. 1991) or from ~ 25 mg of pereopod muscle using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Initially, we amplified COI with the universal primers LCO1490 and HCO2198 (Folmer et al. 1994). However, the PCR product was successfully sequenced only consistently with the LCO primer despite clear amplicons on the agarose gel. An alignment of publicly available mitochondrial genomes of *Eriocheir* showed mutations in the HCO primer region that could have influenced sequencing success. Consequently, a new reverse primer Eri-HCO2198 (5'-TAA ACT TCT GGG TGA CCG AAA AAT CA-3') was designed for amplification and sequencing to improve sequencing success. Samples collected

Table 1. Sampling information.

Country	Sampling location	GPS coordinates	Sampling dates	No. individuals	Sampling site ID	Catalogue no.
Germany	Geesthacht (Elbe River)	53.4261°N, 10.3710°E	23.10.2009	9	EL09	Cr 3207, Cr 3209
			2019–2020	10	EL20	Cr 3687
	Horst (Eider River)	54.3160°N, 9.1901°E	30.11.2009	2	EI09	Cr 3222
			30.4.2020	5	EI20	Cr 3688
	Kiel (Kiel Canal, Kiel Fjord)	54.328751°N–54.3727°N, 9.9641°E–10.1496°E	2008–2009	8	KF09	Cr 3224, Cr 3227, Cr 3201, Cr 3216
		54.3410°N–54.3699°N, 9.7349°E–10.1389°E	2019–2020	21	KF20	Cr 3689, Cr 3690
	Bünzau (Aukrug)	54.0890°N, 9.7970°E	31.07.2008	1	AU08	Cr 3215
	Thedinghausen (Weser River)	52.9828°N–52.9819°N, 9.0272°E–9.0436°E	2019	9	WE19	Cr 3514, Cr 3515, Cr 3516, Cr 3517, Cr 3518
Poland	Vistula Lagoon	54.4657°N, 19.7574°E	10.2020	20	PO20	NA
Belgium	Oostende (North Sea)	51.2281°N, 2.9509°E	08.1998	6	BE98	NTOU B00134
			08.2005	10	BE05	NTOU B00133
	Grobbendonk (Kleine Nete River, Greater Schelde River Basin)	51.1802°N, 4.7390°E	2018–2020	34	BE20	NA
	Doel (Schelde River)	51.3162°N, 4.2676°E	02.09.2020	3	BE20	NA
	Bergenmeersen (Schelde River)	51.02211°N, 3.9645°E	20.10.2020	3	BE20	NA

NA: not available.

in 1998 and 2005 were amplified following the protocol of Xu et al. (2009). For the samples collected between 2008 and 2020, we decreased the PCR mixture volume and lowered the PCR annealing temperature. Each 25 µl amplification reaction consisted of 2 µl DNA template, 1x Roti-Pol TaqS Mix (Carl Roth, Karlsruhe, Germany), 0.5 µM of each primer (Biomers, Germany) and 10 µg bovine serum albumin (Carl Roth). The PCR was carried out at 94 °C for 5 min, then 35 cycles of 95 °C for 30 sec, 47 °C for 60 sec and 72 °C for 60 sec and a final extension at 72 °C for 10 min. The amplified product was sequenced on an Applied Biosystems 3730xl DNA Analyzer in the Institute of Clinical Molecular Biology, Kiel University, Germany (IKMB). We trimmed, aligned and error-checked sequences in Geneious software v. 9.8.1 (Kearse et al. 2012). In addition to the newly-generated COI sequences, we also included the COI data of Hänfling et al. (2002) and the data accumulated by Hayer et al. (2019) for northern, western and central Europe. Otto (2012) reported six additional haplotypes unique to northern Germany that were subsequently included in the study of Hayer et al. (2019) as H12 to H17. We recovered none of these haplotypes, neither in the samples we collected between 2018 and 2020 from the same localities nor in the museum samples that were collected by Thurid Otto herself between 2008 and 2009. These haplotypes are possibly erroneous and all of Otto's sequences were, therefore, excluded from subsequent analysis. Haplotypes were called using the function 'haplotype' of the 'haplotypes' package (Aktas 2015) in the R environment (R Core Team 2019) and a haplotype network was constructed with the function 'parsimnet' of the same package. Haplotypes were labelled following Hayer et al. (2019). We tested for changing proportions of *E. japonica* haplotypes over time using a Pearson's chi-squared test for equality of proportions (function 'prop.test') in the R environment.

For the morphological assessment, we evaluated four presumably species-specific characteristics of the carapace in juvenile and adult mitten crabs that were genotyped at the COI locus: the ratio of carapace width to length, the shape of the infraorbital region, the epi-and protogastric crest and the markedness of the fourth lateral tooth, which were scored as either *E. sinensis*-like or *E. japonica*-like (Guo et al. 1997; Sakai 2013) (Table 2, Fig. 1). We considered crabs adult when they had reached a carapace width of 40 mm, following Peters and Panning (1933). We tested if differences in the character state of the infraorbital region, the crest and the extent of the fourth lateral spine were related to individual size measured as carapace width, sampling locality (Germany vs. Belgium) or COI haplotype (*E. japonica* vs. *E. sinensis*) with a logistic regression. The model was implemented with the function 'glm', specifying the error family as 'binomial' in the R environment.

We conducted a morphometric analysis, based on 27 landmarks (Fig. 1A) on the carapaces of a subset of the genotyped individuals using the 'geomorph' package v. 4.0.1 (Baken et al. 2021) in the R environment. Specifically, we first took pictures of each specimen's carapace using a digital camera, digitised landmarks with the function 'digitize2D', conducted a Generalised Procrustes analysis with the function 'gpagen' and a Principal Component Analysis (PCA) 'gm.prcomp' on the Procrustes-transformed landmark coordinates. We tested for significant ef-

Table 2. Selected species-specific carapace characteristics distinguishing *Eriocheir sinensis* and *Eriocheir japonica* according to Guo et al. (1997) and Sakai (2013). Characteristics are highlighted in Fig. 1.

Trait	*Eriocheir sinensis*	*Eriocheir japonica*
Infraorbital region	With four distinct teeth	Granulated, 4-lobed, separated medially by shallow sinus
4th lateral tooth of carapace	Present	Small or rudimentary, sometimes reduced to a granule
Carapace width : carapace length ratio	1.08 (relatively smaller)	1.12 (relatively larger)
Epi- and protogastric crest	Very strong, high and sharp	Low, weak, blunt

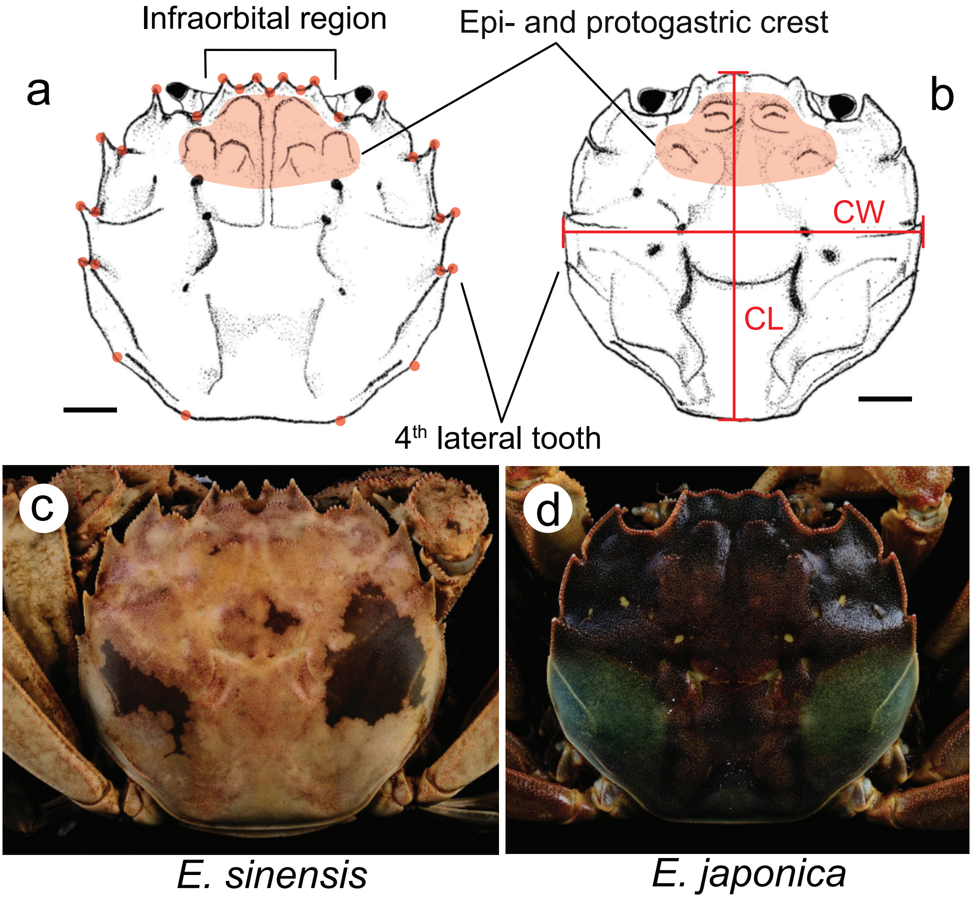

Figure 1. Carapace morphology of the mitten crab species *Eriocheir sinensis* (**A, C**) and *E. japonica* (**B, D**). Line drawings (**A, B**) are reproduced from Sakai (2013) **A** the red dots are the landmarks used in the morphometric analysis **B** the lines indicate where we measured carapace length (CL) and carapace width (CW). The two photographed specimens were collected in China **C** and Japan **D** and deposited at NTOU under voucher numbers B00132 **C** and B00135 **D**.

fects of country of origin, carapace width and their interaction with a Procrustes analysis of variance with 1000 permutations using the function 'procD.lm' of the geomorph package.

Results

A total of 141 specimens were newly sequenced at the COI locus in our study (Table 1). Together with 76 previously-published sequences, the final alignment contained 217 sequences of 511 bp length, from which we identified six haplotypes, five of which had previously been reported from the introduced range (Fig. 2). In addition to the previously reported *E. sinensis* haplotypes H1, H2, H3, H4 and the Japanese mitten crab haplotype H1, two Belgian specimens from 2020 carried the haplotype H18 (Fig. 2), which had only been previously reported from Vladivostok, Russia by Xu et al. (2009).

In total, 38 specimens carried a single *E. japonica* haplotype (Fig. 2), specifically the *E. japonica* haplotype H1 as defined by Hayer et al. (2019). To avoid confusion with the *E. sinensis* haplotype H1, which is also present in Europe, we omit the haplotype designation for *E. japonica* from here on. The mitochondrial DNA (mtDNA) of *E. japonica* was already present in our earliest samples from Belgium collected in 1998, with a frequency of 33% (two out of six specimens, Fig. 3A) and in 50% of the Belgian samples from 2005 (five out of ten specimens). Between 2018 and 2020, 65% of the investigated Belgian crabs had the *E. japonic*a haplotype (26 out of 40 specimens, Fig. 3C). Despite the apparent increase, the proportion of individuals with a *E. japonica* haplotype did not differ significantly between years (Chi-squared = 2.56,

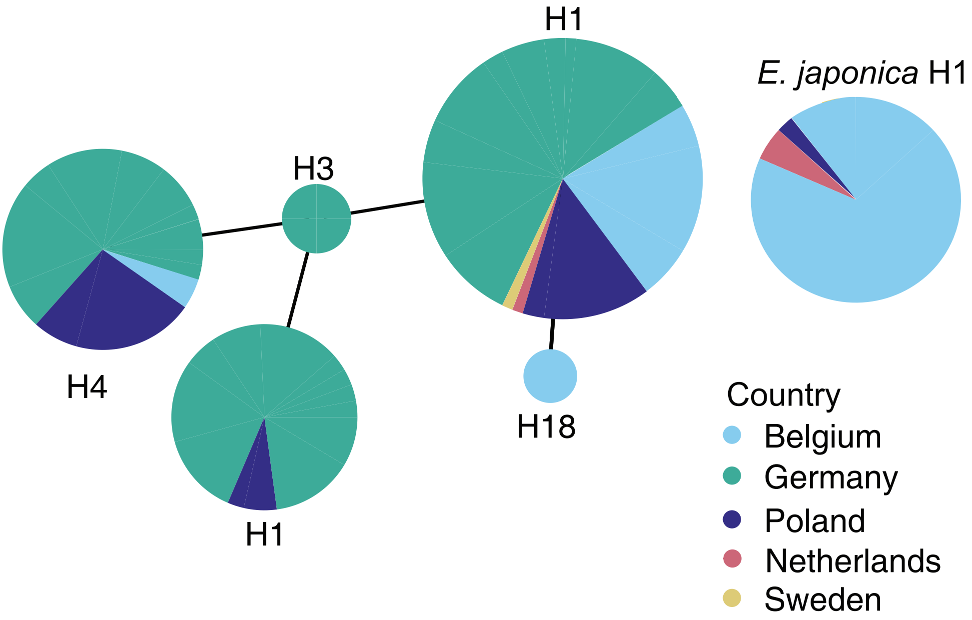

Figure 2. Haplotype network of all analysed sequences from the introduced European range. Each circle represents one haplotype. Size of the circles is proportional to the number of individuals carrying the respective haplotype. Colours denote the sampling locality. The haplotypes were named following Hayer et al. (2019).

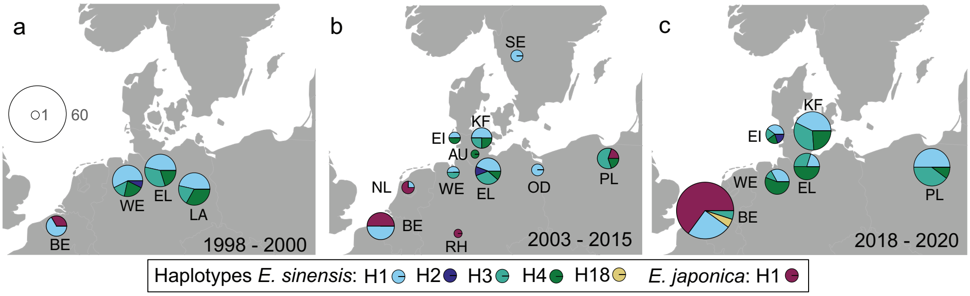

Figure 3. Geographic distribution of COI haplotypes of *E. sinensis* and *E. japonica* in the studied region of Europe, based on newly-generated and publicly-available sequence data **A** the WE (Weser River), EL (Elbe River) and LA (Laascher Lake) populations were sampled by Hänfling et al. (2002) and the BE (Belgium) population was collected in 1998 and used in this study **B** the NL (Netherlands), RH (Rhine River), PL (Poland), OD (Oder River) and SE (Sweden) samples were publicly available and previously synthesised by Hayer et al. (2019). The BE, EI, WE, EL, KF, AU and PL populations were sampled in this study and are detailed in Table 1 **C** all sequences were newly generated in this study and sample information is available in Table 1. Haplotype names correspond to Hayer et al. (2019).

df = 2, p-value = 0.28). Of the Polish samples, 20% (one of five specimens) carried the *E. japonica* haplotype in 2015 (Fig. 3B), but none carried this haplotype in 2020.

All the 41 investigated adult mitten crabs from Belgium, Germany and Poland with a carapace width from 44 to 96 mm were morphologically identified as *E. sinensis*: they had four distinct infraorbital teeth, the fourth lateral spine was pointing outwards, the epi- and protogastric crest was very strong and the carapace length to width ratio was between 1.019 and 1.158 (Suppl. material 1: Table S1, photographs available at 10.6084/m9.figshare.c.5341910).

The 35 examined juvenile crabs from Belgium and Germany were morphologically more variable (Fig. 4A and B). The ratio of carapace width to length was between 1 and 1.157 and, when tested with a linear regression model, did not differ significantly between specimens from Belgium or Germany (std. error = 0.02, t-value = -0.89, p-value = 0.29), specimens with either *E. japonica* or *E. sinensis* COI haplotype (std. error = 0.02, t-value = -0.23, p-value = 0.82) or specimens with different carapace width (std. error = 0.01, t-value = 1.38, p-value = 0.30).

The infraorbital teeth of the juvenile German *E. sinensis* crabs were sharply pointed in all but one individual (ID: KC09-90, Fig. 4C). In contrast, all but one juvenile crab from Belgium had more rounded infraorbital regions (Fig. 4C), without relationship to COI haplotype (std. error = 1.10, z-value = 0.29, p-value = 0.77) or carapace size (std. error = 0.11, z-value = 1.01, p-value = 0.31), based on the results of the logistic regression. Consequently, sampling locality (Germany vs. Belgium) significantly explained the shape of the infraorbital region (std. error = 1.33, z-value = 2.54, p-value = 0.01).

Similarly, the fourth lateral spines pointed outwards in all German individuals, but were less conspicuous in 55% (13 out of 28) of the Belgian individuals (Fig. 4D). The extent of the fourth spine was not related to locality (std. error = 0.01, z-value = –0.01,

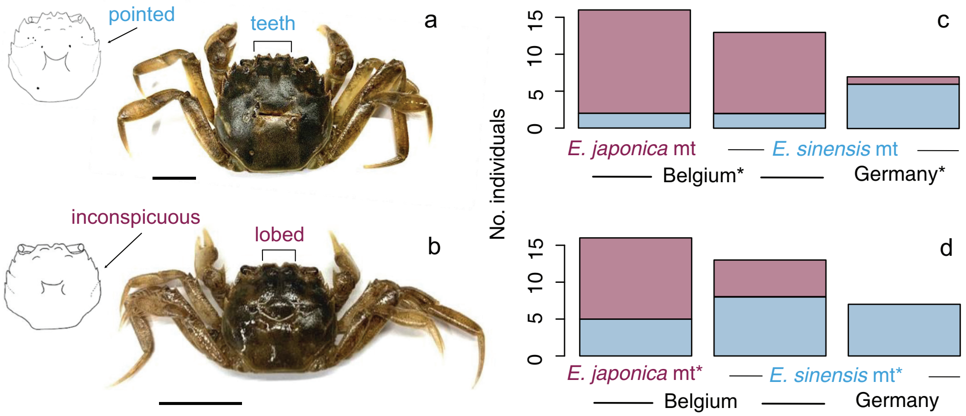

Figure 4. Morphological differentiation amongst juvenile mitten crabs from Germany and Belgium **A** juvenile crab from Germany (ID: KC20-02, collected in the Kiel Canal in 2020, *E. sinensis* mtDNA) with a toothed infraorbital region (brackets) and pointed fourth lateral tooth (arrow) **B** juvenile crab from Belgium (ID: BE20-05, collected in Belgium in 2020, *E. japonica* mtDNA) with a lobed infraorbital region and inconspicuous fourth lateral tooth. Scale bars indicate 1 cm. Distribution of morphological characteristics of infraorbital region **C** and fourth lateral tooth **D** in respect to mtDNA and origin of individuals. Significant differences between groups are marked by asterisks. Purple colour indicates Japanese mitten crab character states and mtDNA and blue colour indicates Chinese mitten crab character states and mtDNA.

p-value = 0.99) or carapace size (std. error = 0.09, z-value = −1.26, p-value = 0.21) in the logistic regression model, but to COI haplotype (*E. sinensis* vs. *E. japonica*) when locality was not included as an explanatory variable into the linear model (std. error = 0.82, z-value = −1.66, p-value = 0.01). The epi- and protogastric crest was strong, high and sharp in all German and most Belgian juveniles. Only four Belgian juveniles had a weakly-defined crest. The expression of this trait was not significantly different between individuals from different countries (std. error = 0.38, z-value = 0.01, p-value = 1.00) or COI haplotypes (std. error = 1.29, z-value = −0.79, p-value = 0.43) and did not vary significantly with carapace width (std. error = 0.13, z-value = −0.37, p-value = 0.71).

The geometric morphometric analysis was conducted on 69 specimens with a carapace width between 15 and 96 mm. Of these specimens, 29 were from Belgium, 27 from Germany and 10 from Poland. In addition, we included three specimens from the native range, two *E. sinensis* from the Yangtze River in China and one *E. japonica* from Shimonoseki in Japan (Fig. 1 shows two of these specimens). The first PCA axis explains 35% of the variability and correlates strongly with carapace width (Fig. 5). The ontogenetic morphological change occurring from juveniles to adults was already described by Peters and Panning (1933), especially with regard to the infraorbital region. In juveniles, the lateral and infraorbital teeth are less pronounced and the intraorbital distance is larger than in adults, which is apparent in the shape of the most extreme specimens in the PCA (Fig. 5A).

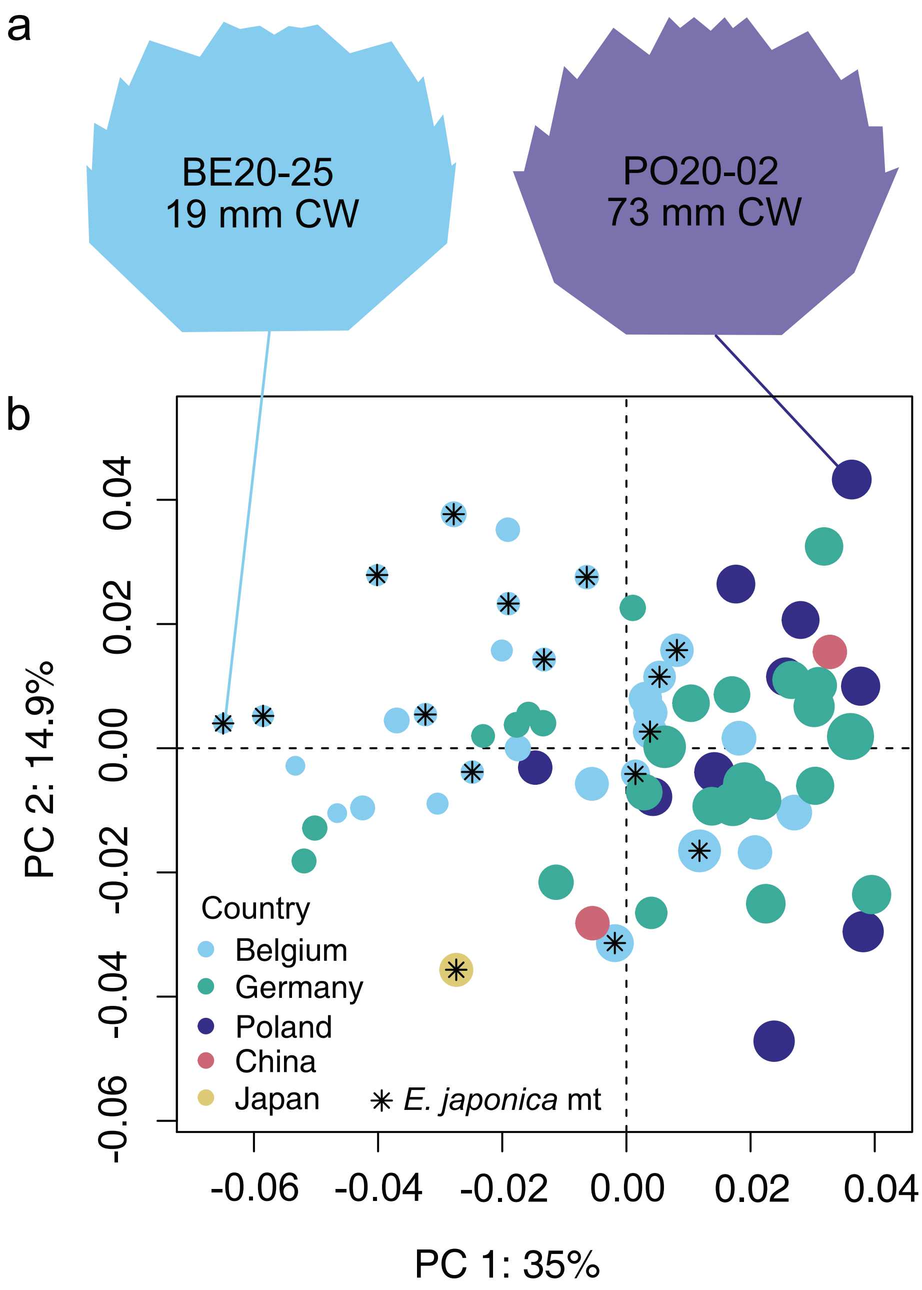

Figure 5. Geometric morphometric analysis on the carapace of mitten crabs from Belgium, Germany and Poland and three native specimens from China and Japan **A** procrustes shapes of two specimens at the opposite ends of the first principal component axis **B** scatterplot of the first two axes of the principal component analysis. Each dot is a specimen, with size proportional to carapace width. Colours denote different sampling countries and asterisks individuals with an *E. japonica* haplotype.

While the PCA does not indicate strong clustering by country, the analysis of variance gave significant results for both carapace width (p-value = 0.001) and locality (p-value = 0.001), but not for their interaction (p-value = 0.12) or mtDNA lineage (*E. sinensis* vs. *E. japonica*) (p-value = 0.16). These results remained the same after removing the native-range specimens from the analysis. The mitten crabs from Belgium, Germany and Poland clustered with the two Chinese mitten crabs from the native range. In the PCA, the Belgian juveniles (about half of which carried *E. japonica* mtDNA) were somewhat distinct from the German juveniles, for which the sample size was small (n = 7).

Discussion

Our study indicates the presence of Japanese mitten crab mtDNA in Belgium since at least 1998, possibly even earlier. Morphologically, however, all adult crabs are identified as Chinese mitten crabs, which explains why the presence of the genetic footprint of Japanese mitten crabs remained overlooked until the employment of molecular analysis. The juvenile crabs from Belgium showed morphological subtle differences to juveniles from Germany, but these differences do not match with the assignment, based on COI haplotypes. This mismatch between morphology and mtDNA implies introgression with a mostly *E. sinensis* nuclear genome, assuming that morphology reflects the nuclear genome well. The increase of *E. japonica* mtDNA from 33% to 65% from 1998 to 2020, observed in Belgian mitten crabs, indicates that this haplotype has become established in Europe and may become more abundant in the region. It likely became established also in the neighbouring Netherlands because the same Japanese mitten crab COI haplotype was found in three out of four mitten crabs collected there in 2011 (Hayer et al. 2019).

Many of the Belgian juveniles show morphological characteristics of *E. japonica*, while most juvenile mitten crabs from Germany show the characteristics of *E. sinensis*. As we did not find the *E. japonica* haplotype in any specimens from Germany or Poland, these populations may not have introgressed, suggesting that juveniles with more or less “pure” *E. sinensis* genomes may differ morphologically from juveniles with an introgressed genomic background. The PCA of the morphometric data also suggests that the morphological differentiation is more pronounced in juveniles. This proposed introgression could have occurred either in the native range, prior to their introduction, or after the introduction of “pure” parental Japanese mitten crabs to Belgium, where Chinese mitten crabs have been present since the 1930s. In support of the first hypothesis, a native hybrid zone exists at the northern limit of the two species’ distribution around Vladivostok in Russia and northern China (Wang et al. 2008, 2013; Xu et al. 2009; Xu and Chu 2012). In this area, over 30% of mitten crabs are hybrids (Xu and Chu 2012).

The region around Vladivostok is a plausible source of the introduction of individuals carrying *E. japonica* mtDNA to Europe, whether pure or hybrid. We identified two individuals from Belgium with the *E. sinensis* haplotype H18 previously only detected in Vladivostok where 3 out of 10 specimens carried this haplotype (Xu et al.

2009; Hayer et al. 2019). The northern range of *E. sinensis* is only represented by Vladivostok in phylogeographic studies, so that we know little about the actual distribution of this haplotype. Nonetheless, Vladivostok itself, with its large port, is not an unlikely source population (Hayer et al. 2019). This could mean that multiple individuals were introduced from Vladivostok (or another locality where the H18 and the introduced *E. japonica* haplotype co-occur) at the same time, at least one with *E. sinensis* mtDNA (the H18 haplotype) and one with *E. japonica* mtDNA. Considering that ballast water was the likely vector, we assume that many larvae with each haplotype could have been introduced. Alternatively, multiple introduction events from different source populations might have occurred into Belgium, with at least one originating from an area where the H18 Chinese mitten crab haplotype occurs, for example, Vladivostok. Under this scenario, "pure" Japanese mitten crabs were introduced to Belgium at a time when Chinese mitten crabs were already present and both species hybridised subsequently in the introduced range. Given the high global shipping traffic to the ports of Belgium, both scenarios are likely. In fact, the scenarios are not mutually exclusive: both introgressed and pure parental species could have been introduced independently. This is alarming as it has resulted in new mitten crab genetic diversity in Europe with unknown effects on the invasiveness of the introgressed specimens.

Interestingly, each of the three European countries shows different temporal dynamics of COI haplotypes. In Belgium, the Japanese mitten crab COI haplotype seems to be increasing over time, though sample sizes are too small to confirm this increase statistically. In northern Germany, the same Chinese mitten crab haplotypes have been present in the past 23 years and the Japanese mitten crab haplotype has never been recorded. In Poland, on the other hand, the specimens sampled in 2020 had a different haplotype distribution to the specimens sampled in 2015. Mitten crabs do not form an established population in Poland where salinity is too low for larval development (Wójcik-Fudalewska and Normant-Saremba 2016). Instead, juveniles and adults may migrate across rivers each year from Germany to enter the eastern Baltic Sea (Czerniejewski et al. 2012). This hypothesis is supported by the haplotype distribution of the 2020 specimens from Poland, which was very similar to the German haplotype distribution. The mitten crabs from Poland sampled in 2015 had a very different haplotype distribution. These crabs may have been accidentally introduced by marine shipping from ports in Belgium and part of The Netherlands or directly from Vladivostok into Russian ports, like Baltiysk or Kaliningrad, located in the Vistula Lagoon. They are testimony to the highly dynamic invasion potential of mitten crabs, apparently due to their high adaptive plasticity (Cui et al. 2021) and explain why Chinese mitten crabs could spread across Europe within decades their initial discovery in Germany in 1912 (Schnakenbeck 1924).

Eriocheir japonica has a similar life history to *E. sinensis* with planktonic larvae and migrating adults, such that similar expansion rates may be assumed (Veldhuizen and Stanish 2002; Dittel and Epifanio 2009; Kalinina 2015). Given the rapid spread of *E. sinensis* within a few decades after its arrival in Europe, it is curious that the *E. japonica* mtDNA has not, to our knowledge, yet spread further than Belgium and The Netherlands. This could indicate that mitten crabs do not disperse between the Belgian and, for example, northern German river systems. Such limited connectivity was already proposed by Peters and Panning (1933), who stated that the Chinese mitten crab remained close to the mouth of a river, even during the planktonic larval phase. The rapid expansion of the Chinese mitten crab in the 1930s across many European countries seems to contradict this scenario, but may be explained by higher levels of anthropogenic dispersal caused by ballast water at that time, for which regulations only recently became stricter (IMO 2004).

Another possible interpretation is that the Belgian population with their introgressed Japanese mitten crab mtDNA has a lower invasive potential. Where the Japanese mitten crab itself has been reported outside its native range, for example, on the west coast of North America (Jensen and Armstrong 2004), the species did not become established. This could be due to a low ability for rapid adaptation, which is likely necessary for successful invasions (Lee 2002). On the other hand, "pure" Japanese mitten crabs are more aggressive than Chinese mitten crabs and win in direct competition for shelter (Zhang et al. 2019). Thus, where "pure" Japanese mitten crabs appear, they may become dominant. However, as the morphology indicates, the Belgian population does not consist of "pure" Japanese mitten crabs, but of Chinese mitten crabs with unknown levels of Japanese mitten crab genomic introgression. Furthermore, it is unclear whether or to what degree the Belgian introgressed population behaves differently from other non-native populations. Future studies should address both the extent of introgression as well as physiological and behavioural differences that could reflect differences in the invasive potential.

There is evidence that the invasion process of mitten crabs is ongoing (Rudnick et al. 2003). In addition to earlier introductions to Europe and the West coast of the United States, *E. sinensis* has colonised Portugal, Spain, Italy and Ireland in the last decades (Cabral and Costa 1999; Rudnick et al. 2003; Garcia-de-Lomas et al. 2010; Crocetta et al. 2020) and has been reported in the Tokyo Bay of Japan (Takeda and Koizumi 2005). *E. japonica* has been reported on the west coast of North America (Jensen and Armstrong 2004) and *E. hepuensis*, native to China, has been introduced to Iraq (Naser et al. 2012) and Iran (Naderloo 2014). These new findings warn us that mitten crabs are still on the move. The role of introgression for the invasive potential in this species has not been considered. Given the fact that *E. sinensis* is a recognised global invasive species (e.g. it has been included in the list of invasive alien species of European Union concern; European Commission 2016), understanding the ecological consequences of this introgression and its geographic extent might contribute for the development of an effective control and management strategy.

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Supplementary material 1

Table S1. Master spreadsheet with morphological and genetic information for each specimen

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RESEARCH ARTICLE

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A review of volunteers' motivations to monitor and control invasive alien species

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Abstract

People make an important contribution to the study and management of biological invasions, as many monitoring and control projects rely heavily on volunteer assistance. Understanding the reasons why people participate in such projects is critical for successful recruitment and retention of volunteers. We used a meta-synthesis approach to extract, analyze and synthesize the available information from 28 selected studies investigating motivations of volunteers to engage in monitoring and control of invasive alien species (IAS). Our findings show how motivations fit three broad themes, reflecting environmental concerns, social motivations, and personal reasons. An important outcome of this study is the description of motivations that are unique to the IAS context: supporting IAS management, protecting native species and habitats, and livelihood/food/income protection or opportunities. In addition, our study reflects on important methodological choices for investigating volunteer motivations as well as ethical issues that may arise in practice. We conclude with a set of recommendations for project design and future research on volunteer motivations in IAS contexts, emphasizing the importance of collaboration with social scientists.

Keywords
Biological invasions, biodiversity monitoring, citizen science, perceptions, public engagement, social dimensions

Introduction

Public involvement in the monitoring and control of invasive alien species (IAS) contributes to both increased scientific understanding and effective management of biological invasions in multiple ways (Hester and Cacho 2017; Roy et al. 2018, Larson et al. 2020a; Pawson et al. 2020; Price-Jones et al. 2022). Species occurrence data collected by volunteers can improve our understanding of IAS distributions and inform modeling of species range expansion (Brown et al. 2008; Bois et al. 2011; Gallo and Waitt 2011; Crall et al. 2015; Grason et al. 2018; César de Sá et al. 2019; Giovos et al. 2019; Lehtiniemi et al. 2020). People can also play an important role in the early detection of IAS (Looney et al. 2016; Carnegie and Nahrung 2019; Epanchin-Niell et al. 2021). For example, in New Zealand, 63% of detections of new pest incursions were attributed to the general public (Bleach 2018 in Epanchin-Niell et al. 2021), while in the United States, the general public and private owners of nurseries and farms detected 27% of new alien pests found between 2010 and 2018, including a large number of species with high economic or environmental impact (Epanchin-Niell et al. 2021). People can also play active roles in the capture, control and removal of IAS (Bryce et al. 2011; Kobori et al. 2016; Marchante and Marchante 2016; Dechoum et al. 2019; Jubase et al. 2021). Additional benefits of engaging people in IAS projects include increased public awareness of IAS (Jordan et al. 2011), potentially resulting in the prevention of new introductions (Azevedo-Santos et al. 2015), changed behavior which can reduce the spread of IAS (Cole et al. 2016, 2019), and wider acceptance and support of IAS control and eradication (Larson et al. 2016; Novoa et al. 2017; Dunn et al. 2018, 2021; Bailey et al. 2020; Phillips et al. 2021).

Understanding volunteer motivations is critical for effective volunteer recruitment, retention, and the long-term sustainability of volunteer-driven projects (Wright et al. 2015; Cardoso et al. 2017; Veeckman et al. 2019; Rüfenacht et al. 2021). Different theories have been proposed to explain why people spend time and effort on volunteer tasks (see West and Pateman 2016 for a recent synthesis). Such motivations may be intrinsic, meaning that a person finds fulfillment in the volunteer work itself (e.g. through learning or altruistic concerns), or extrinsic when it offers external rewards (e.g. increased job prospects) (Finkelstein 2009). Previous research in the field of social psychology notes that “acts of volunteerism that appear to be quite similar on the surface may reflect markedly different underlying motivational processes” (Clary et al. 1998, p. 1517) and posits that motivations of individuals may be derived from a person’s values (i.e. finding it important to help others), the drive for understanding and knowledge (i.e. wanting to learn), building and maintaining social connections and capital (i.e. strengthening relationships or sense of community), career perspectives (i.e. gaining career-related experience), self-protection (i.e. reducing negative feelings), or personal development (e.g. growing or developing psychologically) (Clary et al. 1998; Clary and Snyder 1999; Omoto and Packard 2016).

Research on environmental volunteering, including volunteer motivations, gained traction in the last two decades, especially in countries with a long tradition in people's involvement in biodiversity monitoring, such as the United Kingdom, the Netherlands, Australia and the United States (Measham and Barnett 2008; Geoghegan et al. 2016; Merenlender et al. 2016; Ganzevoort 2021), or countries with a long history of managing IAS, such as South Africa (Shackleton et al. 2019; Jubase et al. 2021). Previous studies have empirically tested and classified different motivations in an environmental context (Bruyere and Rappe 2007; Measham and Barnett 2008; Larson et al. 2020b). For example, Measham and Barnett (2008) present a set of six broad motivations underpinning environmental volunteering (i.e. contributing to community, social interaction, personal development, learning about the environment, a general ethic of care for the environment, and attachment to a particular place or species) and five different modes through which volunteering is manifested (i.e. activism, education, monitoring, restoration, and promoting sustainable living). Large scale surveys among environmental volunteers have shown that they can have multiple reasons for participating, and that motivations vary by socio-demographic attributes and the type and extent of participant involvement (Ganzevoort and van den Born 2020; Larson et al. 2020b).

One environmental area in which participation of volunteers is increasing relates to biological invasions (Johnson et al. 2020; Price-Jones et al. 2022). The ability to purposefully design projects for monitoring and controlling IAS in which the public is involved requires knowledge of the motivations of individuals to participate in such initiatives (Hobbs and White 2012; Roy et al. 2018; Pocock et al. 2020; Encarnação et al. 2021). While a number of studies have synthesized research on perceptions of IAS (Kapitza et al. 2019; Shackleton et al. 2019; Cordeiro et al. 2020), we do not know of any research which does this relating to volunteer motivations. To fill this gap, our primary objective was to synthesize existing knowledge about the diverse motivations of volunteers who participate in IAS monitoring (e.g. citizen science initiatives) and control projects (i.e. hands-on activities to manage IAS). Although these two types of activities are different, they are closely linked as monitoring or observing IAS often contributes to decisions about management actions. This connection is sometimes very clear, for example in early detection and rapid response (de Groot et al. 2020), but there are also more implicit ways in which monitoring data informs decision-making in IAS management and science (Groom et al. 2019). In this paper, we simply refer to 'IAS projects', including both monitoring and control activities, but as motivations may differ for the two, we emphasize important differences when they arise.

To achieve our objective, we used a meta-synthesis approach to extract, analyze and synthesize the available information about volunteer motivations from relevant scientific and grey literature. This approach is useful for analyzing a relatively small number of studies on a selected topic (Hoon 2013) and is increasingly applied in the context of environmental and other interdisciplinary studies (Carlson and Palmer 2016; Garavan et al. 2019).

Although initially our synthesis focused on documenting and better understanding the diversity in volunteer motivations, while conducting our meta-synthesis, we further identified important methodological and practical implications of study and project design. First, we found large differences in how and to what extent studies investigated motivations, ranging from very limited quantitative reports to in-depth qualitative inquiries. This led us to document the different approaches and methodologies that were used for measuring volunteer motivations and to what extent they were reported in the articles. Second, we also paid close attention to ethical and practical dilemmas reported in the studies. At the end of the paper, we provide a number of recommendations for (i) designing projects that consider the diverse motivations of participants to maximize recruitment and retention, and (ii) future research on volunteer motivations in IAS contexts.

Methods

Bibliographic analysis

We searched for relevant publications using multiple databases and sources for peer-reviewed and grey literature. A bibliographic search was conducted using both Web of Science (WoS) on February 10, 2021 and SCOPUS on March 5, 2021. The search string captured three main topics (i.e. motivations, citizen science and volunteering, and invasive alien species) and we included multiple synonyms for each topic: (motivation* OR engag* OR incentive*) AND ("citizen science" OR volunteer* OR community) AND ("invasive species" OR "alien species" OR "exotic species" OR "non-native" OR "nonnative" OR "non-indigenous" OR invas*). We used the filtering options of the databases to exclude publications from other fields (e.g. healthcare, physics). This search resulted in a list of 267 bibliographic references in WoS and 302 in SCOPUS. Next, we scrutinized the title and abstract and, if needed, the full text of the articles, to further exclude articles that did not relate to IAS or did not contain any information pertaining to volunteer motivations to participate in IAS projects. Combining the searches from WoS and SCOPUS databases, we selected 18 relevant articles (of which six were found using SCOPUS, but not included in WoS).

Additionally, we conducted a search using the Google and Google Scholar search engines using (variations of) the same search string and reviewing the first 50 results, as relevant search results declined quickly and were not found in the last 30–50 results. This yielded three additional references. We also requested information from working group leaders of the EU Cooperation in Science and Technology (COST) Action AlienCSI (www.alien-csi.eu) via email, obtaining one additional unpublished dataset (Marchante et al., pers. comm.) and one recently published paper. Finally, we included five additional papers obtained via the snowball sampling strategy, i.e. by retrieving relevant papers from the reference lists of the selected papers.

Altogether, 28 sources were selected for inclusion in the meta-synthesis. Despite using different search strategies covering academic and non-academic literature, we did not find any grey literature sources. This may be a limitation of our search being in English only, as such reports are likely to be published in local languages.

Data analysis

Each of the selected papers was read in full by the first and last author who made extensive notes about the study context, methodologies and findings. First, we documented the specific context of each study using the questions and categories in Table 1. Second, we listed each phrase or text fragment referring to motivations (hereafter 'motivation statement') that was presented in the paper. A more detailed explanation of how we categorized these motivations is given below. Third, we collected detailed information about the methodology and/or approach that was used for measuring motivations using the questions and categories in Table 2. Finally, we systematically listed important findings or recommendations that linked motivations to the design and evaluation of IAS volunteer projects. These findings were grouped and summarized according to specific themes.

Table 1. Questions and categories used for describing study contexts.

Name	Question	Categories
Year	In which year was the paper published?	Free text
Country	In which country did the study take place?	Free text
Volunteer type	Which type of volunteers were involved in the project?	IAS project volunteers*, specific target audiences (e.g. landowner, hunter, divers, etc.), the general public, or other
Project type	What was the main aim of the project in which volunteers participated?	Control, detection/monitoring or other
Target species	What was the target species?	Free text
Target species group	To what species group does the species belong?	Bird, fish, insect, mammal, plant, reptile or other
Habitat type	Which habitats did the study cover?	Terrestrial, freshwater, marine, or island

* i.e. volunteers already involved in an ongoing IAS project

Table 2. Questions and categories used for describing study methodologies for measuring motivations.

Name	Question	Categories
Data collection	What was the main method used for data collection?	Questionnaires, interviews, both or other
Respondent number	How many respondents answered the question about motivations?	Free text (number)
Question type	What type of question was used to measure motivations?	Open, closed, both or other
Documentation	Did the study provide adequate information about the data collection method and questions (either in the main text or in an appendix)?	Yes or no

Classification of motivations for participating in IAS projects

We collated a list of 233 motivation statements retrieved from the 28 papers (See Suppl. material 1). The listed motivations were assigned to broader categories using iterative coding (c.f. Asah et al. 2014). The iterative coding process was both deductive (i.e. based on previously known motivation categories; Measham and Barnett 2008; Wright et al. 2015; West and Pateman 2016; Larson et al. 2020b) and inductive (i.e. by identifying and grouping motivations that did not resemble previously known motivations categories and assigning them to new categories). Visualization of the conceptual framework was done using open source diagrams.net software (https://www.diagrams.net/).

Some statements included multiple motivations, for example, when the participants expressed both an attachment to a particular place and a more general desire to help the environment, or a wish to contribute to science, while experiencing fun and enjoyment at the same time. In such cases, the motivation statement was assigned to multiple categories, thus resulting in a higher number of recorded motivations than the total number of recorded statements (264 vs. 233, respectively). While a number of motivation statements were assigned to preexisting motivation categories (e.g. contribution to science, helping the environment, social interaction, attachment to a particular place, or wanting to share existing knowledge with others), others required us to develop a new set of categories unique to volunteers participating in IAS projects.

To decrease subjective interpretations, the categorization was performed by a team of four researchers. The first author developed the initial categorization scheme and started the process of ascribing motivations to appropriate categories. Three of the co-authors joined the process of categorization by providing their own views on the appropriate categories, thus ensuring that the final result of the categorization was not influenced solely by the perspective of one author. Motivations which were categorized differently were discussed until consensus was reached.

Results and discussion

Study contexts

All papers included in the analysis (See Suppl. material 1) were published in the last ten years (2012–2021) except for one study (Krasny and Lee 2002). Most studies were conducted in Europe (n = 12) and North America (n = 9), with three conducted in Australia and one study each in South Africa, the Caribbean, Hawaii and the Canary Islands. Studies reporting on terrestrial ecosystems (n = 24) strongly outnumbered those related to marine environments (n = 4). Majority of the studies (n = 20) reported volunteer motivations for participating in control projects only, while six studies reported on monitoring projects and one each on the training of IAS monitoring and management planning. The projects often targeted a specific IAS and these were mostly plant (n =

11) and mammal (n = 5) species, or both (n = 1), while the remaining were focused on invasive fish (n = 3), birds (n = 3), reptiles (n = 2), insects and tree insect and fungal pest species (one study each). The one remaining study did not specify any IAS target group. The reviewed studies investigated the motivations of groups of volunteers committed to participating in IAS projects (n = 14), or specific target audiences (e.g. land-owners, hunters, divers, etc.; n = 11), while the remaining studies focused on the general public (n = 3) or the participants of a training program on invasive species (n = 1)

Motivations

Iterative categorization of the 233 motivation statements resulted in 15 different motivations affecting the participation of volunteers in IAS projects (Table 3). The conceptual framework presented in Fig. 1 shows how these motivations fit three broad themes, reflecting primarily (1) environmental concerns (i.e. supporting IAS management, helping the environment, and protecting native species and habitats), (2) social motivations (i.e. social interaction, community responsibility, and contribution to science), and, (3) personal reasons (i.e. learning something new, personal and career development, feeling of accomplishment, health and wellbeing, and enjoyment and fun). A number of motivations belong to more than one theme. For example, contact with nature and attachment to a particular place are motivations which include both a personal and an environmental aspect, while the desire to share existing knowledge and livelihood/food/income protection or opportunities are influenced by the social and personal aspect of motivations (Fig. 1).

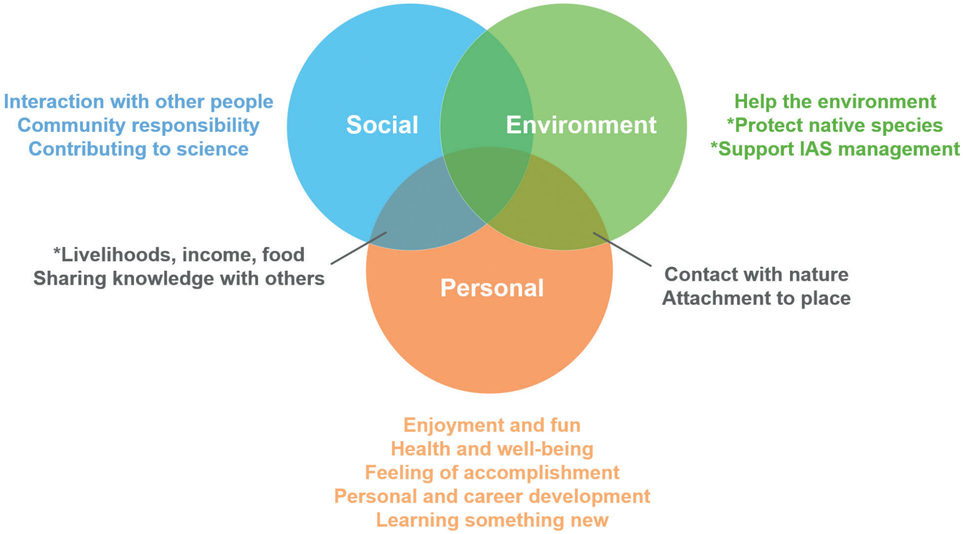

Figure 1. Conceptual framework for understanding volunteer motivations for participating in monitoring and control of invasive alien species along three main themes. Newly identified motivations unique to the IAS context (compared to existing literature) are marked with an asterisk.

Three motivations unique to the IAS context emerged during the analysis: 1) supporting IAS management (Environment theme), 2) protecting native species and habitats (Environment theme), and 3) livelihood/food/income protection or opportunities (Social/Personal theme). We describe these motivations in more detail in the section on "*Motivations specific to IAS context*". In addition, eleven motivations were described in previous literature (Clary and Snyder 1999; Measham and Barnett 2008; Finkelstein 2009; West and Pateman 2016; Larson et al. 2020b). Eight statements could not be categorized and were termed 'Other' (Table 3). Five of these statements were related to previous experience of participating in citizen science initiatives (Marchante et al. 2017; Garrard et al. 2020; Jubase et al. 2021; Phillips et al. 2021; Marchante et al. pers. comm.) which led people to take part in subsequent activities. The remaining three were fear of the species itself (Servia et al. 2020), "desire to preserve environmental aesthetics" (Jubase et al. 2021, p. 4), and cost, with participation in the project being "cheaper than a normal biology course or dive" (Cerrano et al. 2017, p. 316).

Motivations specific to IAS context

As stated above, three motivations unique to the IAS context emerged (Table 3). Supporting IAS management emerged as a leading motivation of volunteer participation (expressed through 30 statements in 20 papers, Table 3). This category groups motivations that start from an understanding of the harmfulness or alien status of IAS, leading to a desire to assist in their management/eradication. While some simply expressed a desire to remove the IAS "to aid conservation management" (Stien and Hausner 2018, p. 189) or for the "chance of keeping them [tree pests or diseases] at bay or eradicating them" (Pocock et al. 2020, p. 724), others recognized that IAS are not meant to be in the introduced area (e.g. "carp don't belong here and there are too many of them" in Atchison et al. (2017, p. 340)).

Ten papers outlined that participants were motivated primarily by the wish to protect native species and habitats in their surroundings (e.g. Harvey et al. 2016; Niemiec et al. 2016; Cerri et al. 2018; Crowley et al. 2018; Pagès et al. 2018, 2019; Dunn et al. 2021). This motivation reflects the close relationship, affection, and attachment people feel towards native species (e.g. red squirrels in Crowley et al. (2018); puffins in Pagès et al. (2018)) and habitats (e.g. native broadleaved woodlands in Crowley et al. (2018)), and consequently their desire to protect these from the impacts of IAS. Often respondents reported that they feel that native species are more valuable than alien species (Pagès et al. 2019) and that humans are responsible for the introduction of alien species, making it our moral duty to control them (Crowley et al. 2018). Similarly, the study of Jubase et al. (2021) also reports this motivation, expressed as a desire to preserve the unique native fynbos biome in South Africa.

Livelihood/food/income protection or opportunities refers to the protection or improvement of livelihoods and incomes in cases where IAS cause crop damages (e.g. Cerri et al. 2018; Saavedra and Medina 2020) or have a negative effect on business

Table 3. Motivations of volunteers participating in invasive alien species monitoring and management projects (categories unique/specific for IAS contexts are given in bold).

Theme	Motivation	Description	Examples	Reported In*:
Environment	**Supporting IAS management**	A desire to participate in IAS control and eradication	see Section "*Motivations specific to IAS context*"	[3], [4], [7], [8], [9], [10], [11], [12], [13], [14], [16], [17], [18], [19], [20], [21], [22], [23], [24], [26], [27], [28]
	Helping the environment	A desire to help the environment	"help nature" "protect the environment" "assist with conservation efforts"	[1], [3], [4], [5], [7], [9], [10], [11], [13], [14], [16], [17], [18], [19], [21], [22], [28]
	Protecting native species and habitats	A desire to protect native species and habitats	see Section "*Motivations specific to IAS context*"	[4], [6], [8], [9], [11], [12], [16], [20], [21], [22]
Environment/ Personal	Contact with nature	Opportunity to experience nature, being in close contact with the natural world, unique experiences in nature	"opportunity to work in close contact with the natural world" "opportunity to experience impressive nature"	[1], [2], [5], [10], [12], [16], [18], [21], [22], [23], [28]
	Attachment to a particular place	Feeling of attachment to local places	"personal attachment to local places" "taking care of favourite dive sites"	[1], [5], [9], [22]
Personal	Learning something new	A general interest in acquiring new knowledge	"to learn more about the environment/IAS" "learning something new"	[1], [2], [4], [5], [7], [10], [12], [13], [14], [18], [21], [28]
	Personal/career development	Interest in acquiring new skills; education, or career progression	"learning job skills" / "skill development" "gaining additional field experience" "use of novel technologies"	[1], [2], [4], [5], [7], [10], [14], [17], [18], [21], [22]
	Enjoyment/fun	Expressions of positive emotions like enjoyment and fun	"thrill seeking" "exciting experience"	[1], [2], [3], [4], [5], [12], [13], [15], [16], [23], [28]
	Health and wellbeing	References to mental and physical health	"to get out of the house" "to get exercise" "a good form of relaxation"	[1], [2], [9], [13], [21], [22], [28]
	Feeling of accomplishment	Feelings of pride, satisfaction and doing something that is meaningful	"to show that I can make a difference" "I feel I'm doing it right and I am proud"	[1], [2], [5], [12], [18], [19]
Personal/Social	**Livelihood/food/ income protection or opportunities**	References to protecting or improving livelihoods and incomes	see Section "*Motivations specific to IAS context*"	[2], [3], [4], [6], [8], [11], [19], [20], [22], [25], [27], [28]
	Wanting to share existing knowledge	Wish to share existing knowledge with others	"to let children/ grandchildren know the sea" "to teach others about invasive species"	[2], [3], [5], [13], [14], [18]

Theme	Motivation	Description	Examples	Reported In*:
Social	Contribution to science	Wish to contribute to or take part in scientific research	"because data can be useful for science" "participation in exciting discoveries"	[5], [15], [17], [18]
	Community responsibility	Feeling a sense of responsibility / duty of care / giving something back to the community	"for the future generations" "showing that one can make a difference" "moral duty to manage the consequences"	[1], [2], [3], [4], [5], [6], [8], [12], [13], [16], [18], [19], [21], [22], [24]
	Social interaction	Spending time with friends, family, or like-minded people	"spending time with friends/family" "being with people that share interests" "a sense of belonging to a group"	[1], [2], [4], [5], [9], [12], [13], [14], [18], [21], [22], [28]

* The numbers in brackets refer to the number in the List of studies used in the analysis, provided in Suppl. material 1.

profitability and property value (e.g. Marshall et al. 2016; Pagès et al. 2019). This motivation also includes IAS as a new source of food or income (e.g. Carballo-Cárdenas and Tobi 2016; Atchison et al. 2017), where local communities have recognized the potential of either eating a particular IAS, or by selling products and services related to the target IAS (e.g. lionfish in Carballo-Cárdenas and Tobi (2016)).

Observed differences in motivations

Our meta-synthesis indicated some minor (and expected) differences in motivations between volunteers participating in either detection/monitoring or control projects. Participants in control projects did not report being motivated by 'contributing to science', while participants in detection/monitoring projects did not report 'protecting native species/habitats' or 'health and wellbeing' as motivations. However, these findings are based on a limited number of studies and most of these were linked to control projects. In order to provide more insights, we need comparative study designs measuring the types and strength of motivations in different kinds of projects.

We observed some interesting patterns of motivations for different target groups. Land-owners and local residents (Marshall et al. 2016; Niemiec et al. 2016; Saavedra and Medina 2020; Dunn et al. 2021) have a vested interest in their own neighborhoods or properties, are more locally oriented, and therefore motivated by the desire to protect their livelihood/food/income opportunities, develop social interactions with their neighbors and contribute to their community. Hunters (Stien and Hausner 2018) and divers (Carballo-Cárdenas and Tobi 2016; Cerrano et al. 2017) are often motivated by the opportunity for fun and enjoyment, outdoor recreation/sport, and contact with nature. Additionally, divers reported an attachment to a particular place (e.g. a preference for certain diving spots; Cerrano et al. 2017), a desire to contribute to science, share knowledge and develop personally/career-wise.

Pagès et al. (2019) observed differences in motivations within groups of project volunteers controlling the same IAS, ranging from helping nature to protecting private property, or seeing the IAS as threatening to their recreational activities. They also found differences in motivations between groups of volunteers controlling different target IAS (i.e. grey squirrel vs. Himalayan balsam). The most notable difference was that while supporting IAS management was seen as the leading motivation in the group of volunteers controlling Himalayan balsam, those tasked with killing invasive grey squirrels saw it as a disincentive for participation, rather than motivation (a more detailed discussion on the ethical problems of killing animals is made in the section on "*Ethical and practical dilemmas*"). In other studies, the participants taking part in the control of invasive mammals (e.g. squirrels; Crowley et al. 2018; Dunn et al. 2021) and reptiles (e.g. Burmese pythons; Harvey et al. 2016) were motivated to protect native species and habitats.

Methodologies and approaches used for measuring motivations

The majority of the studies (n = 17) used online or paper questionnaires for data collection, five studies conducted interviews, three studies used both questionnaires and interviews, and two were based on participant observations. Generally, the questionnaires included closed questions (e.g. multiple choice, ranking). Interestingly, very few of the questionnaire studies draw upon existing typologies from social science literature (the exception being Asah and Blahna 2012; Asah et al. 2014 who adopt a functionalist perspective), pointing to an obvious research gap as well as a lack of scholarly exchange between disciplines. Interviews with open questions gathering qualitative data provided more novel insights, which informed our section on 'new motivations' in the section *"Motivations specific to IAS context"*. Less than half of the studies (n = 12) provided a copy of their questionnaire as supplementary material, or adequately explained their methods in the text. Our synthesis approach did not answer questions of relative importance of motivations, or directly compare outcomes from different studies. This was difficult due to the great diversity in methods used to measure motivations, lack of reporting on methodological procedures and outcomes, and large differences in target groups and sample sizes.

Initial and sustained motivation: changes over time

A number of studies, mainly related to IAS control, investigated temporal dimensions of motivations, by measuring them at several points in time. For example, in their study of volunteers in urban conservation via invasive plant control, Asah and Blahna (2012) found that social and personal benefits were better predictors of the frequency of participation than more often reported environmental-related reasons. In a similar vein, Carballo-Cárdenas and Tobi (2016) reported that participant motivations shifted from collective reasoning (i.e. to help the environment) to individualistic reasoning, including promoting commercial and recreational harvesting of lionfish. In this case, sustained interest was thus mainly driven by self-interest. This confirms findings from previous research that self-reported motivations (often measured at the start of a project) are not necessarily influential motivations that predict the duration of the engagement (Ryan et al. 2001).

Pagès et al. (2018) studied volunteers' initial and sustained motivations by surveying and interviewing prospective, new, returning, experienced and inactive volunteers in invasive plant management on islands. They found that motivations changed from identifiable functional reasons to start volunteering (i.e. personal goals/circumstances and project aims) to more complex attachments to the place and group over time. Similar was observed by Jubase et al. (2021), who have shown that in 43% of volunteers a difference was observed between the initial reasoning for getting involved in IAS management and the motivations to remain involved in these activities. This implies that experiences during volunteer activities influence motivations, however, this change in motivation can be both positive and negative. Creating unique experiences for participants, e.g. by visiting places which are otherwise off limits can be an incentive to participate and can also result in a greater sense of responsibility for the volunteer or better relations between volunteers, stakeholders and management authorities based on trust (Cerrano et al. 2017; Pagès et al. 2018).

Another study noted that the perceptions of control feasibility can shift initially optimistic views to more nuanced, realistic or even pessimistic perspectives on the effectiveness of IAS control (Pagès et al. 2019). This, in turn, can affect volunteer retention, as participants may become disengaged over time, doubting or questioning the value of what they are doing (Atchison et al 2017). One way of dealing with this specific issue is to design the project in such a way that volunteers can see tangible results and feel like they are making a difference, e.g. by first clearing a field of weeds mechanically before bringing in volunteers to remove the last remaining plants (Pagès et al. 2019) or observing the recovery of indigenous vegetation (Jubase et al. 2021). Early detection of species is a rare event which may also reduce motivation to participate in monitoring (Pocock et al. 2020). In such cases, it is important to carefully communicate about the species' detectability to foster realistic expectations and avoid backlash where people's participation results in reduced concern about IAS because they cannot find it (Falk et al. 2016; Harvey et al. 2016).

Ethical and practical dilemmas

Motivations of project organizers and volunteers can differ substantially, leading to practical and ethical dilemmas. Pocock et al. (2020) give some clear examples from the context of early detection of tree pests and diseases and how this may affect motivations of participants to join or stay engaged in citizen science projects. One dilemma is that local communities can be disproportionately affected by actions following detection of pests and diseases as negative impacts, while the benefits of these actions are more likely gained at a larger scale. People may stop reporting due to concerns about the impacts of eradication measures, both due to the method used (e.g. killing, pesticides) or their outcome (e.g. felling trees or restricting access, sometimes resulting in a loss of income). Thus, in contrast to their expectation of helping the environment (e.g. to save trees), participants' efforts in reporting may lead to unintended consequences (e.g. as saving trees involves felling some of them). Similarly, Pagès et al. (2019) point to the potential failure in reconciling multiple goals of participation (e.g. gathering more data vs. empowering people) and warn that an overemphasis on conservation and cost-effectiveness criteria can fail to address local communities' concerns.

Another issue is that volunteers may be regarded as an answer to labor shortages and escalating costs. Some of the studies reported that detecting and monitoring species in marine environments is relatively expensive. Engaging volunteers can reduce costs for working hours and equipment, but resources can also be a constraint for volunteers to participate (Carballo-Cárdenas and Tobi 2016). In general, citizen science and volunteering should not be regarded as free labor of any kind, as it may actually result in higher associated costs for stakeholders or organizations (e.g. due to the high workload in confirming observations, communication or training of volunteers).

Awareness of volunteer perceptions is especially important when their activities involve or contribute to the killing of animals. Studies report that this can be an emotional burden on people, especially with charismatic invasive animal species such as grey squirrels or Asian carp (Atchison et al. 2017; Crowley et al. 2018; Dunn et al. 2021). Killing invasive animals brings combined ethical and practical constraints. For example, even in large scale invasive animal control projects (involving hundreds of thousands of animals being killed, e.g. Bonnet et al. 2021), the majority of volunteers may not have access to the most humane methods of dispatch, forcing them to rely on a blow to the head or to drowning the target animals (e.g. Crowley et al. 2018). Olszańska et al. (2016) have shown that such methods received lower support or were even opposed by the public, making it vitally important to communicate, promote and make the most humane methods for killing the target IAS more accessible, as they are often either not known or not practiced by a broader audience (e.g. Atchison et al. 2017). The discourse of ethics should not be confined solely to methods used for killing animals. Rather, it also needs to consider the potentially negative impacts on the wellbeing (both health and safety) of volunteers and the social implications (e.g. being regarded as animal killers) for people who volunteer in such eradication campaigns.

A final dilemma concerns the decision to reward volunteers or not. Several studies report on the risk of crowding out intrinsic motivations if authorities promote personal benefits such as financial rewards (Stien and Hausner 2018; Garrard et al. 2020). On the other hand, it may promote inclusiveness by enabling participation of diverse volunteers that could have financial barriers (Pateman et al. 2021). The examples we found were linked to (semi)professional hunters receiving a reward per animal. As a rule, the other volunteer activities were unpaid, even though it is important to highlight that reimbursements can be important in enabling volunteer participation.

Recommendations for future research

Our synthesis revealed that studies of volunteer motivations in IAS contexts are often pragmatic without making reference to theoretical frameworks. Inadequate reporting of methods was another issue. Our findings also point towards the importance of considering situation-specific drivers and temporal changes when measuring motivations. In addition to scoring or ranking motivations, it is thus also important to test whether such self-reported motivations actually influence behaviors and whether they change over time. Such longitudinal and explanatory studies remain scarce within the context of volunteer projects and require more attention. Differences in motivations between and within specific volunteer groups highlight the need to understand the target group of volunteers. This would ensure better success in their recruitment and retention throughout volunteer projects. Our recommendations for future research are to:

- Design survey methods that build upon previous research on motivations, behavior and knowledge from different research disciplines (e.g. Clary and Snyder 1999; Wilson 2000; Omoto and Packard 2016).
- Include demographic information in the study for possible segmentation of the sample into different groups with different motivations, e.g. nationality, gender, age, income, level of education, ethnicity, disability status and employment status.
- Comply with transparency and FAIR data policies, e.g. publish questionnaires used, results and other relevant methodological information as standard practice.
- Use comparative study designs for measuring the types and strengths of motivations in different kinds of projects or comparing between different groups.
- Test whether self-reported motivations align with observed behavior and whether these change over time (longitudinal and explanatory studies).

Recommendations for designing future volunteer projects

Most papers provided concrete recommendations for designing projects that consider the diverse motivations of participants to maximize their recruitment and retention (See Suppl. material 3). For example, it is important to be inclusive of diverse groups of people and tailor tasks or roles to their interests and capabilities (MacLeod and Scott 2021; Pateman et al. 2021). To this end, providing clear information on what is required from the volunteers, how much time would they need to invest and what support they can expect from the project is important. Also, projects should consider active recruitment strategies based on information from potential participants regarding their preferences for reporting data or contributing to IAS management. Some concrete recommendations for designing IAS volunteer projects based on the information reviewed in our work (See Suppl. material 3) and our personal experiences are:

1. Document and report participant demographics (age, gender, participant profile, etc.) to monitor diversity in citizen science, evaluate engagement and devise strategies to improve inclusiveness.
2. Consider whether volunteers can have a larger role in co-designing or collaboratively developing the project. Asking (potential) volunteers about their needs and wishes before and during a project enables the targeting of specific audiences and adapting to their needs.
3. Organize activities that provide volunteers with unique opportunities, exciting experiences, and fun and enjoyment.

4. Visit locations where (potential) volunteers can see and experience the negative impacts of IAS first-hand. Such an experience can trigger a desire to help or to continue volunteer activities.
5. In case of control projects, clearly state the management objectives of projects to avoid disillusionment.
6. Carefully consider ethical, legal and financial aspects around the involvement of volunteers, particularly in control projects. Provide adequate support to volunteer work that involves high risk activities (e.g. insurance).
7. If possible, partner up with existing projects and initiatives to ease volunteer recruitment and avoid ‘competition’ between projects.
8. Promote long-term projects that allow for continuity and for “knowing and recognizing the brand”.
9. Promote collaboration between different stakeholders, e.g. between the government and volunteer organizations.
10. Ensure that information about the programs is made more accessible (due to problems with internet access, and social media platform usage).

Conclusions

Knowledge of volunteer motivations is important for developing and improving project design, communication, and evaluation of IAS projects. Despite increasing public involvement in monitoring and control of IAS, our synthesis found that only a limited number of studies have investigated volunteer motivations to participate in such activities. Our conceptual framework identified 15 motivations of which three were unique to the IAS context: supporting IAS management, protecting native species and habitats, and livelihood/food/income protection or opportunities. This framework, including environmental, social and personal motivations, provides a clear starting point for developing survey instruments, though the selection and number of survey items will depend on the target audience. We encourage researchers and project managers to amplify their efforts in systematically gathering and reporting data on participant motivations in IAS projects, to allow for comparative studies and quantitative assessments of the importance of certain motivations. Collaboration with social scientists is strongly recommended to ensure the use of appropriate methodologies and consideration of relevant theoretical frameworks.

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Supplementary material 1

List of the studies used in the analysis

Authors: Ana A. Anđelković, Lori Lawson Handley, Elizabete Marchante, Tim Adriaens, Peter M.J. Brown, Elena Tricarico, Laura N.H. Verbrugge

Data type: pdf file

Link: https://doi.org/10.3897/neobiota.73.79636.suppl1

Supplementary material 2

Overview of the study characteristics and methodological approaches of the selected papers

Authors: Ana A. Anđelković, Lori Lawson Handley, Elizabete Marchante, Tim Adriaens, Peter M.J. Brown, Elena Tricarico, Laura N.H. Verbrugge

Data type: pdf file

Link: https://doi.org/10.3897/neobiota.73.79636.suppl2

Supplementary material 3

List of recommendations for designing projects to ensure maximum recruitment and volunteer retention extracted from the studies used in the analysis (for their full references, please see Suppl. material 1) and their link to our recommendations

Authors: Ana A. Anđelković, Lori Lawson Handley, Elizabete Marchante, Tim Adriaens, Peter M.J. Brown, Elena Tricarico, Laura N.H. Verbrugge

Data type: pdf file

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Combining surveys and on-line searching volumes to analyze public awareness about invasive alien species: a case study with the invasive Asian yellow-legged hornet (*Vespa velutina*) in Italy

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Abstract

The Asian yellow-legged hornet (*Vespa velutina*) has been invading Italy since 2013, and it was subjected to management programs to counter its spread and raise awareness about its impacts. We administered a questionnaire to a sample of 358 beekeepers in Italy, asking them about their sources of information on *V. velutina* and their perception of its potential impacts and severity, compared to other threats to beekeeping. We also explored Internet searching volumes on Google and Wikipedia about *V. velutina*, to identify seasonal and long-term trends in public awareness. Workshops, journals or bulletins, the Internet and word-of-mouth with colleagues were the primary sources of information about *V. velutina*. Internet searches peaked during the activity period of the species and increased over time, with thousands of visits to Wikipedia each month. Beekeepers believed *V. velutina* to affect beekeeping by predating on honey bees (*Apis mellifera*), paralyzing foraging, reducing honey availability and depleting the winter cluster. Moreover, *V. velutina* has been deemed a severe threat to beekeeping, similar to other threats like pesticides and the varroa mite (*Varroa destructor*). Our findings indicate that beekeepers seem to be aware of the potential impacts of *V. velutina* in Italy, both within and outside of its invaded area, considering it a major threat to beekeeping. Moreover, citizens seem to have become progressively curious about the species. Information campaigns on the Internet and specialized magazines might be helpful to communicate about the impacts of the species, and the need to develop diffused surveillance networks.

Keywords
Beekeeping, biological invasions, Google, Google Trends, species management, Wikipedia

Introduction

Biological invasions are a major driver of change globally, with environmental and socio-economic impacts, whose frequency and magnitude are increasing in synergy with international trade and climate change (Hulme 2009; Robinson et al. 2020). Therefore, many countries have developed public policies aimed at preventing, counteracting, or mitigating biological invasions (Turbelin et al. 2017), including both dedicated legal frameworks and also financial support for specific conservation projects targeting invasive alien species (e.g. the LIFE programme in the European Union, European Commission Directorate-General for Environment 2015).

Most conservation projects include outreach activities to raise the awareness of specific stakeholders, and/or the general public about biological invasions and invasive alien species (Lioy et al. 2019a). Awareness raising is a prerequisite for attitudinal and behavioral change, which could in turn foster compliance with regulations about biological invasions or the long-term endorsement of dedicated policies (Heberlein 2012). Surveys based on questionnaires are a common approach to measure awareness about biological invasions. Before-and-after designs (Novoa et al. 2017) and repeated cross-sectional designs (Russell 2014) are commonly adopted to test for temporal changes in public awareness. However, while these studies might be effective for specific stakeholders or over small spatial scales, they might fail to consider broader changes in public awareness that occur at larger spatial and temporal levels of detail. For example, while a before-and-after survey could measure changes in public awareness following a project about a particular invasive alien species, the same species might be targeted by multiple projects over a time span of several years. Designing a specific survey for this scope might be unfeasible and expensive.

On the other hand, at a time where the Internet is becoming a primary source of information worldwide, many studies showed that a growing number of people search on the Internet for those topics they are curious about, or have heard about, from various sources of information. Therefore, the analysis of online searching volumes on search engines and Wikipedia could become a valuable tool to measure public interest towards political (Mellon 2013, 2014) as well as environmental issues (Nghiem et al. 2016; Burivalova et al. 2018), including biological invasions (Cerri et al. 2020; Cerri et al. 2022). In this study, we aimed to show that it is possible to combine questionnaire-based surveys with the analysis of Internet searching volumes to draw conclusions about the awareness of stakeholders and the general public on invasive alien species by considering the case of the invasive Asian yellow-legged hornet (*Vespa velutina* Lepeletier, 1836) in Italy.

V. velutina has become invasive in Europe, where it was first reported in 2004 in France, and is increasing its distribution across Central and Mediterranean countries and in the UK (Laurino et al. 2020). From 2013 onwards, several nests of the species have been reported in Italy, where it colonized the westernmost portion of the Liguria region, close to the French border, from which it then expanded to some areas of the Piedmont and Tuscany regions (Bertolino et al. 2016; Lioy et al. 2019b).

Apart from its impact on native insects and wild pollinators, its intensive predation upon the western honey bee (*Apis mellifera*), its reproductive potential and the lack of specialized predators, *V. velutina* can have severe impacts on beekeeping in Europe (Laurino et al. 2020). The predation of honey bees could decrease the pollination services they provide, undermine honey production and reduce the availability of individuals for the winter cluster, with consequences for the overwinter survival of the colonies (Monceau et al. 2013). The predator activity of *V. velutina* also limits the foraging activity of honey bees by promoting homing failure and determining a "foraging paralysis", where honey bees do not leave the colony fearing its predation (Requier et al. 2019), which could further reduce pollination services and honey production. *V. velutina* could also damage fruit production, as adult individuals need sweet carbohydrates to sustain their metabolism. Finally, *V. velutina*, by building nests mainly on artificial structures or trees in or near urban areas, can also be problematic due to the risk of stings to people (de Haro et al. 2010) that in some cases could lead to fatalities caused by anaphylactic reactions to stings (Feás Sánchez and Charles 2019).

The management of invasive *V. velutina* is becoming an important issue for some European countries, with France documenting an expenditure of about €23 million for nest removal in the 2006–2015 period (Barbet-Massin et al. 2020), while Spanish beekeepers from la Coruña province reported an expenditure of about €67,000 just for 2016 (Ferreira Golpe et al. 2016). The hypothetical cost for managing the species if it were to colonize all the climatically suitable areas in France, Italy, and the UK is estimated to reach about €29.5 million per year (Barbet-Massin et al. 2020).

Due to its potential impacts on beekeeping, and its associated costs, the invasion of *V. velutina* has been targeted by various conservation projects in Italy. These include the LIFE STOPVESPA (https://www.vespavelutina.eu) and the LIFE ASAP (https://www.lifeasap.eu) projects, the Aliem Interreg Med project (http://interreg-maritime.eu/web/aliem), all three projects co-founded by the European Union, and the projects VELUTINA and STOPVELUTINA (https://www.stopvelutina.it/il-progetto). While these initiatives differed in their spatial scale and specific goals, all of them included many outreach initiatives about *V. velutina*, such as press campaigns, meetings with stakeholders and workshops at beekeeping events, with the main intention of raising the awareness of both beekeepers and laypeople.

The aim of the present study was to investigate public awareness about the invasive hornet *V. velutina* in Italy by implementing two different approaches for analyzing i) how beekeepers perceived the impact of the species, and ii) how online searches on *V. velutina* varied over time. Therefore, we first administered a questionnaire to a sample of beekeepers in Italy to ask them about their perception of *V. velutina* and its impact (also in relation to the other threats affecting honey bees) and their primary sources of information about the species. Then, we conducted a time-series analysis to identify long-term trends in on-line searches on Google and Wikipedia about *V. velutina* in Italy to capture temporal trends in public awareness about the species.

Methods

Questionnaire design and administration

In August 2019, we designed a questionnaire on GoogleForms, to measure beekeepers' perception of *V. velutina* as a threat to beekeeping and the conservation of honey bees. The questionnaire was divided into four different sections for measuring: i) the primary sources of information about *V. velutina* adopted by the beekeepers, ii) the perceptions about the most significant impacts of *V. velutina* on beekeeping and human activities, iii) the severity of *V. velutina* as a threat to beekeeping, compared to other major threats, and iv) the characteristics of respondents and their beekeeping activity.

Information sources were evaluated by asking respondents to complete a check-box with some of the main types of traditional and digital media: the Internet, newspapers, television or radio, specialized magazines, beekeeping bulletins, social networks, mailing lists, word-of-mouth with other beekeepers, communication with agronomists or entomologists, beekeeping workshops and significant beekeeping events (e.g. showrooms, conventions).

The main impacts of *V. velutina* that we mentioned in the questionnaire included a reduction in honey production caused by predation on honey bees and the inhibition of foraging, decrease in honey bees for the winter cluster, disease transmission to honey bees by foraging upon multiple colonies, damages to fruit orchards, increased risk of stings for the beekeepers. Moreover, we asked whether the impact of *V. velutina* was greater than that of the native European hornet (*Vespa crabro*). We asked for respondents' agreement with a series of statements about these impacts on a 5-point bipolar scale, ranging from "Totally disagree" to "Totally agree". As respondents may not have been familiar with some of the impacts, questions also had an "I don't know" option.

Then we asked respondents about which were the main threats to beekeeping, in their opinion. These included honey bee predation by *V. velutina*, predation by native Hymenoptera, predation by birds, pesticide poisoning, infestation by the small hive beetle (*Aethina tumida*), infestation from the varroa mite (*Varroa destructor*), nosemosis or fungal, bacterial and viral diseases. Each of these threats was evaluated on a 5-point unipolar scale, ranging from "Not serious at all" to "Extremely serious".

In the final section, we asked respondents whether they came from an area that had already been invaded by *V. velutina*, the decade when they started beekeeping, the size of their apiary, their sex, age and level of education, as well as the location (at the district level) where they practice beekeeping.

Questionnaires were forwarded to a sample of beekeepers in Italy, both from invaded and non-invaded areas, through a snowballing approach. Researchers who already operated in the management of *V. velutina* contacted referents from beekeeping organizations, asking them to forward the questionnaire to their contacts. This approach was chosen because a representative sample was not achievable with other techniques due to the absence of complete data about single beekeepers and the impossibility of designing a sampling strategy to recruit them in the field because of their different habits and the spatial scale of the study. A complete copy of the questionnaire in English and Italian language is available in Suppl. material 1.

Analysis of Google Trends and Wikipedia data

To measure whether there was an increase in public awareness about *V. velutina* through time, in Italy, we explored the temporal evolution of the volume of searches on Google about the Italian name for the species "*Calabrone asiatico*" (literally, Asian hornet, in Italian) and also the scientific name "*Vespa velutina*", which has become widely adopted. Moreover, we also explored the temporal evolution in the monthly number of visits to the Wikipedia page "*Vespa velutina*" since 2015.

Google Trends is a relative index obtained by dividing the total number of searches related to a specific query by the total volume of searches on Google. The index is then rescaled between 0 and 100 by assigning the maximum value (100) to the point of the time series with the highest value of the index. Therefore, Google Trends is a relative metric, which is strongly discounted for the increasing number of searches on Google over time. On the other hand, the WikiMedia foundation allows users to access the number of visits, expressed as a raw count, to the various pages of Wikipedia, at least since July 2015. The combined use of Google Trends and Wikipedia visits therefore enabled us to both identify whether searches for *V. velutina* had become more common through time, as well as to appreciate their order of magnitude.

Data analysis

To highlight differences in beliefs about the impacts of *V. velutina* between respondents from the invaded and the non-invaded range of the species, as well as in its perception as a threat to beekeeping, we compared the distribution of answers using the Potential for Conflict Index (PCI, Vaske 2018) and a chi-square test of independence. To be consistent with analysis of Google Trends data, we adopted a Bayesian implementation of the chi-square test, based on the Bayes factor. The Bayes factor essentially assess the odds of the alternative hypothesis of an association between two variables, over the null hypothesis of independence. We ran the Bayesian test of association with the BayesFactor package (Morey and Rouder 2021) using default priors and Poisson sampling plan, as the number of subjects had not been fixed in advance. The PCI is a common measure of respondents' polarization in human dimensions studies adopted in surveys with bipolar or unipolar scales, ranging between 0 and 1. The minimum value of the PCI indicates the maximum agreement between respondents, when their answers lie entirely on the same point of the scale, while the PCI peaks when respondents are equally divided between the two opposite points of the scale. Moreover, we tried to segmentate respondents according to their sources of information about *V. velutina*, through a hierarchical cluster analysis.

Online searches on Google, based on the Google Trends index, were converted on a logarithmic scale, then decomposed in their long-term trend and their seasonal component, based on Bayesian structural time series with a Gaussian distribution of the error, a state-space model for time-series data (Scott and Varian 2013). Statistical analyses were carried out with the statistical software R (R Core Team 2020).

Data resources

Datasets and reproducible software code are available at https://osf.io/efs7k/.

Results

Structured questionnaire

Overall, we collected 358 surveys from our sample of beekeepers. Most respondents (59.7%) came from Liguria, Piedmont and Tuscany, regions that had already been invaded by *V. velutina*, while the remaining beekeepers from uninvaded regions, almost entirely in Central and Northern Italy (Fig. 1). Most respondents were men (82.3%), with a higher education (86.0%) and an age between 36 and 65 years (18–25 years = 2.8%, 26–35 years = 13.4%, 36–45 years = 28.2%, 46–55 years = 27.4%, 56–65 years = 17.0%, over 65 years = 11.2%). The majority of respondents started beekeeping after 2010 (62.0%) or in the early 2000s (16.5%) and had a small apiary (5 colonies or fewer = 26.8%, 6–10 colonies = 27.7%, 11–25 colonies = 25.1%, 26–50 colonies = 8.9%, 51–100 colonies = 5.6%, more than 100 colonies = 5.9%).

Beekeepers apprised themselves about *V. velutina* from multiple sources of information, in particular beekeeping workshops (53.4%), specialized journals (51.4%), the Internet (49.2%), beekeeping bulletins (42.5%), word-of-mouth with other beekeepers (37.7%) and agronomists/entomologists (36.3%), beekeeping events (20.9%), social networks (19.3%), generalist newspapers (17.6%), television or radio shows (14.0%) and mailing lists (6.1%). Hierarchical cluster analysis did not identify any major cluster of respondents, but instead 10–11 small clusters whose differences were unclear.

Most respondents believed *V. velutina* to have major impacts on honey bee colonies, mostly by reducing honey production through bee predation and foraging paralysis, and by decreasing the size of winter clusters. Moreover, respondents believed that *V. velutina* could increase the risk of stings to beekeepers and that its impacts were more severe than those of the native European hornet (*V. crabro*) (Fig. 2). On the other hand, respondents were less certain about the potential role of *V. velutina* in disease transmission to honey bees by foraging over multiple colonies.

Respondents from invaded areas were more certain than those from uninvaded areas that *V. velutina* represents a severe threat to honey bees because of its foraging behavior (Bayes factor (*BF*) for the test of association of 116:1). The *BF* indicated that the odds of the alternative hypothesis of an association between these two variables was 116 times more likely than the null hypothesis of no association. For the other impacts, no differences were observed between respondents from the invaded and uninvaded range (BF_{10}: honey availability = 0.04; winter cluster = 0.47; fruit production = 0.04; disease transmission = 0.25; risk of stings = 0.08; European hornet = 0.73).

V. velutina was considered a severe threat to honey bees and beekeeping. Respondents from invaded areas assigned it a severity score comparable to pesticides or the varroa mite (*V. destructor*) (Fig. 3). Moreover, respondents from the invaded areas were

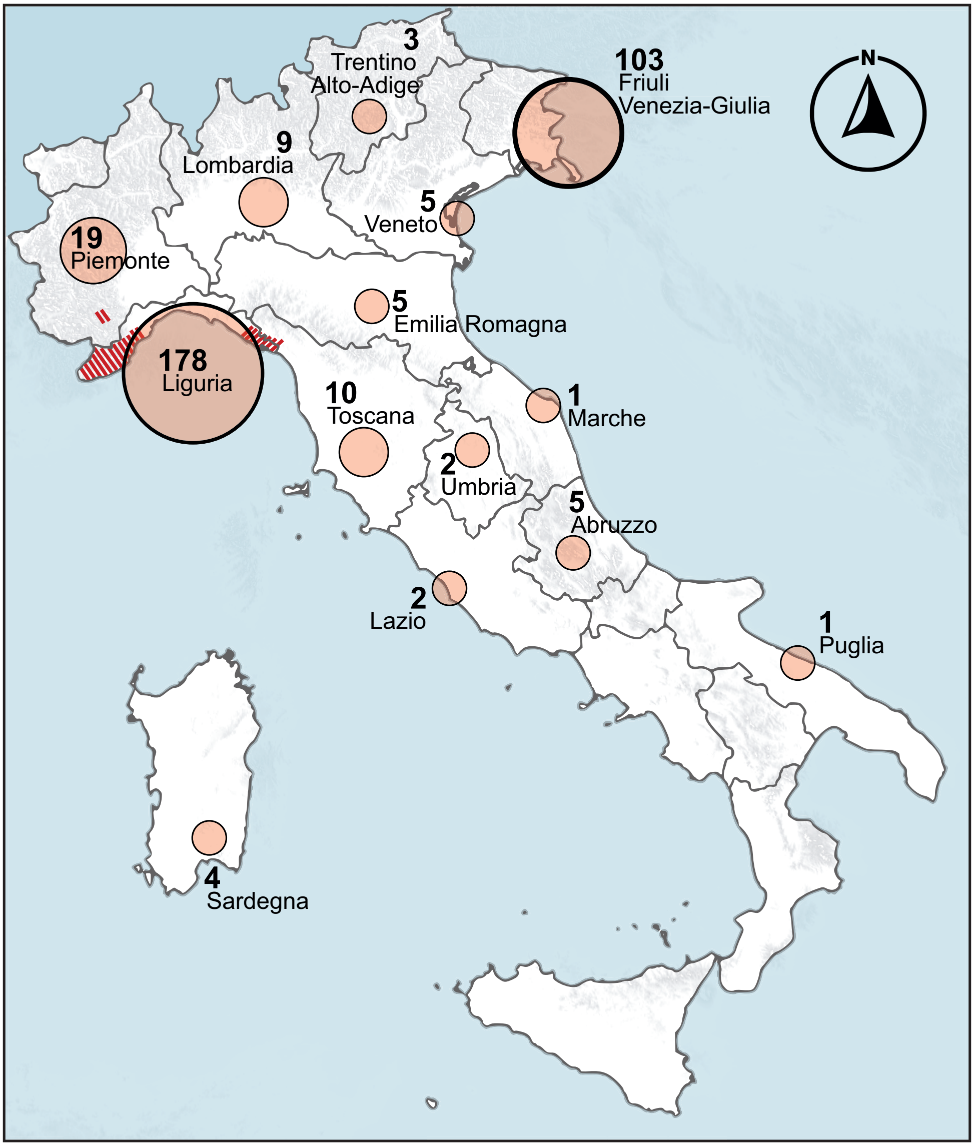
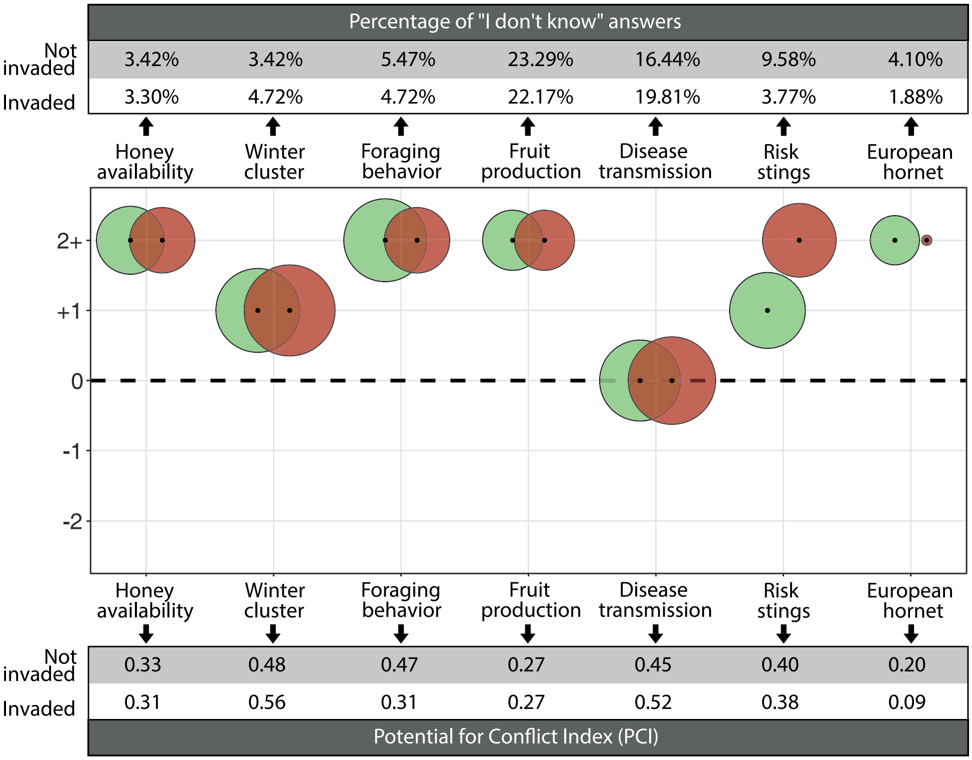


Figure 1. Geographical distribution of respondents between the various Italian regions. Dashed areas correspond to the invaded range of *V. velutina* in Italy, in the Piemonte, Liguria and Toscana regions. Numbers above region names correspond to the number of respondents in each region. Circle areas were assigned arbitrarily to represent differences in the number of respondents between regions. 11 respondents did not indicate their region.

more certain that *V. velutina* poses a serious threat to honey bees than respondents from uninvaded areas (BF_{10} = 26). For the other threats to beekeeping, no differences were observed between respondents from the invaded and uninvaded range (BF_{10}: pesticides = 0.002; native Hymenoptera = 0.15; birds = 1.47; *Aethina tumida* = 0.10; bacterial diseases = 0.05; *Nosema apis* = 0.08; fungal diseases = 0.03; viral diseases = 0.03;



Percentage of "I don't know" answers	Honey availability	Winter cluster	Foraging behavior	Fruit production	Disease transmission	Risk stings	European hornet
Not invaded	3.42%	3.42%	5.47%	23.29%	16.44%	9.58%	4.10%
Invaded	3.30%	4.72%	4.72%	22.17%	19.81%	3.77%	1.88%

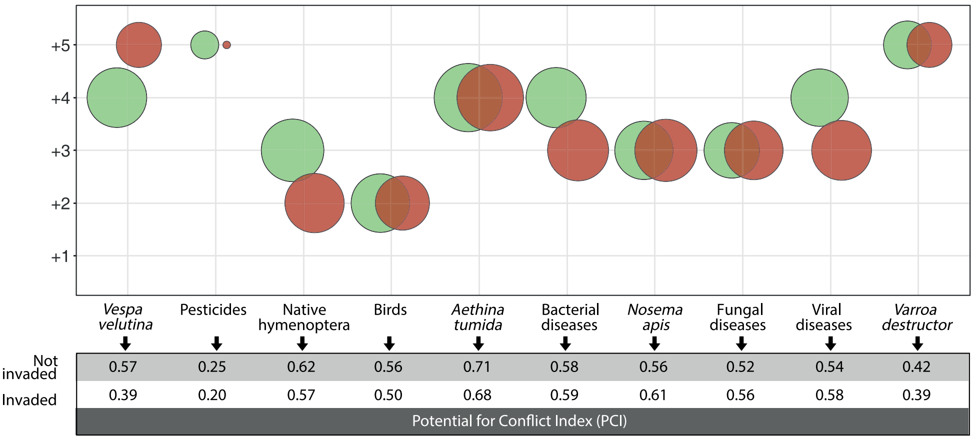
Potential for Conflict Index (PCI)	Honey availability	Winter cluster	Foraging behavior	Fruit production	Disease transmission	Risk stings	European hornet
Not invaded	0.33	0.48	0.47	0.27	0.45	0.40	0.20
Invaded	0.31	0.56	0.31	0.27	0.52	0.38	0.09

Figure 2. Perceived impacts of *V. velutina*. Comparisons of the perceived impacts between respondents from the invaded and the non-invaded range of the species. Answers were measured on 5-points of a bipolar scale (y-axis), ranging from "Strongly disagree" (-2) to "Strongly agree" (+2). Bubbles were centered on the median score of invaded and non-invaded areas, and their size was proportional to the Potential for Conflict Index, which ranged between 0 (no disagreement, all answers on the same point of the scale) and 1 (respondents were equally divided between the two opposite points of the scale). Bubbles on the left (in green) represented answers from respondents living in non-invaded areas, while bubbles on the right (in red) answers from respondents living in invaded areas.

Varroa destructor = 0.03). The distribution of scores to the questions on the perceived impacts of *V. velutina* and its severity in relation to various threats for beekeeping is available in the Suppl. material 2.

Analysis of Internet searching volumes

The Google Trends index for the queries "*Calabrone asiatico*" and "*Vespa velutina*" showed clear seasonal fluctuations, with a high number of searches between April and October, corresponding to the activity period of *V. velutina*. Searches usually had two peaks per activity period. Moreover, the two queries had an increasing long-term trend in their number of searches on Google. Notably, while the volume of searches for "*Vespa velutina*" mostly increased until summer 2015 and then stabilized, the query "*Calabrone asiatico*" increased steadily through time (Fig. 4).



	Vespa velutina	Pesticides	Native hymenoptera	Birds	*Aethina tumida*	Bacterial diseases	*Nosema apis*	Fungal diseases	Viral diseases	*Varroa destructor*
Not invaded	0.57	0.25	0.62	0.56	0.71	0.58	0.56	0.52	0.54	0.42
Invaded	0.39	0.20	0.57	0.50	0.68	0.59	0.61	0.56	0.58	0.39

Figure 3. Perceived severity of various threats for beekeeping. Comparisons between respondents from the invaded and the non-invaded range of the species. Answers were provided on a unipolar scale ranging from "Not at all serious" (+1) to "Extremely serious" (+5) (y-axis). Bubbles were centered on the median score of each answer, and their size was proportional to the Potential for Conflict Index, which ranged between 0 (no disagreement, all answers on the same point of the scale) and 1 (respondents were equally divided between the two opposite points of the scale). Bubbles on the left (in green) represented answers from respondents living in non-invaded areas, while bubbles on the right (in red) represented answers from respondents living in invaded areas.

The monthly number of visits to the Italian Wikipedia page for *V. velutina* was quite high and variable (median ± sd = 2503 ± 3042), but it showed a similar seasonal pattern, with visits increasing between April and October and being characterized by a double peak in this timespan (Fig. 5).

Discussion

To the best of our knowledge, this study constituted a first attempt to draw conclusions about the awareness of beekeepers and citizens towards *V. velutina* in an invaded area of Europe. While another study (Requier et al. 2020) explored the behavior of beekeepers in response to *V. velutina*, no study formally asked beekeepers about their beliefs on the potential impacts of *V. velutina*, or about its potential magnitude with respect to other threats that could affect honey bees and beekeeping activity. Taken together, findings from the questionnaire for the beekeepers and the analysis of Internet searching volumes, indicate that public awareness increased over the years after the detection of the species in Italy, and that stakeholders are aware about the invasion of *V. velutina* and its social and ecological impacts. The different conservation projects developed in Italy over the years may have contributed to achieving these results, together with dissemination activities by beekeeper associations and by the media in general.

Beekeepers in Central and Northern Italy seem to have received considerable exposure to news concerning *V. velutina* and its potential environmental and socio-economic

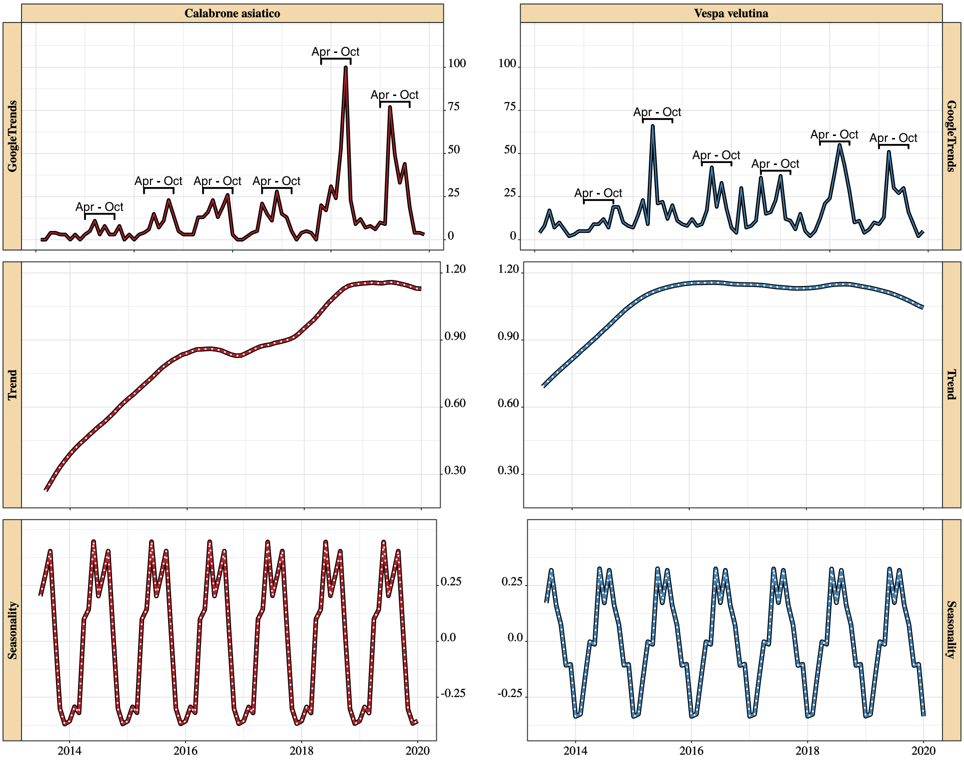


Figure 4. Volume of searches on Google. Volume of searches for the queries "*Calabrone asiatico*" and "*Vespa velutina*", the two Italian words mostly used for naming the species. Google Trends index (top), long-term trend (center) and seasonal component (low) of the log-converted Google Trends index.

impacts. Such exposure, in turn, affected their concerns about the species. The vast majority of our sample believed that *V. velutina* could have detrimental impacts on the conservation of honey bees and beekeeping, in line with the available scientific evidence (Requier et al. 2019; Laurino et al. 2020). Respondents from invaded areas seemed to be even more concerned about this. They believed *V. velutina* to have greater impacts than the native *V. crabro*, another species that could prey on honey bees. Moreover, these impacts were also considered relevant for beekeeping and the conservation of honey bees. *V. velutina* severity was considered comparable to that of other major causes of honey bee decline, such as pesticides (Sánchez-Bayo et al. 2016; Tsvetkov et al. 2017) or *Varroa destructor* (Thoms et al. 2019), and even more severe than other stressors, such as the predation from other native Hymenoptera, or viral, fungal and bacterial diseases. While our findings came from a convenient sample of beekeepers, which may have been more in contact with beekeeping organizations and more aware than the "average" beekeeper, we believe our findings to be strong enough that it is unlikely that the scenario from the overall beekeeping community is very much different. *V. velutina* seems to be considered a species with systematic and not-negligible impacts on honey bees and beekeeping.

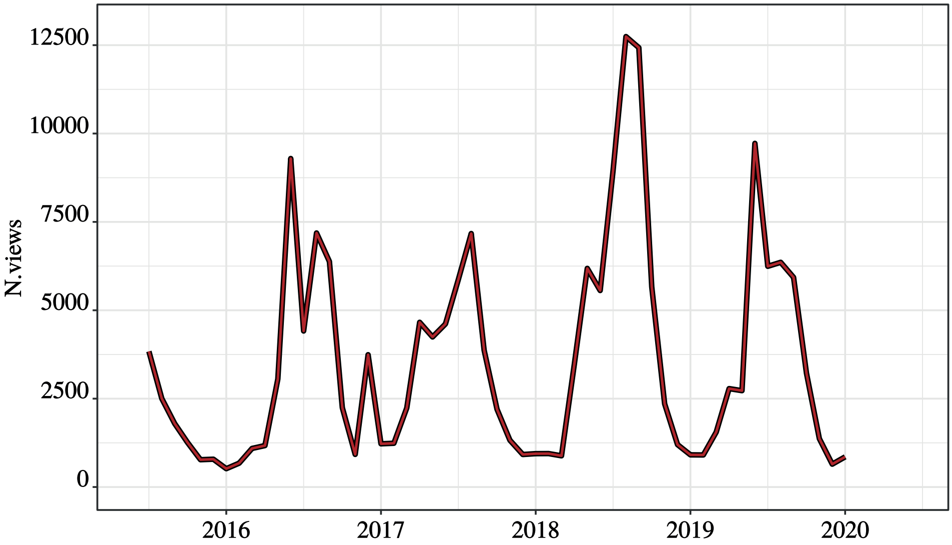


Figure 5. Volume of searches on Wikipedia. Temporal evolution of the monthly number of visits to the Wikipedia page "*Vespa velutina*", in Italy, over the last few years. The time series starts in July 2015.

Moreover, we found that beekeepers apprised themselves about *V. velutina* from a wide range of different channels, encompassing both the Internet and specialized magazines, and also activities with other members of their community, like other beekeepers and professionals holding workshops. On the other hand, conventional media and mailing lists seemed to be minor information sources on *V. velutina*.

These findings might help to design communication campaigns about *V. velutina* among beekeepers. The fact that beekeepers seem to be at least familiar with the species and its impacts indicate that they might have stable attitudes about this topic (Heberlein 2012). Their involvement in ongoing management initiatives confirms this. Both LIFE STOPVESPA and STOPVELUTINA projects have seen the participation of many beekeepers in the monitoring of *V. velutina* distribution (see projects website). The awareness of the impact caused by *V. velutina* and the willingness to collaborate in its management is fundamental for the extension at the national scale of an Early Warning and Rapid Response system already developed in the invaded area (Porporato 2016). Considering the sources of information that are adopted the most by beekeepers, conservationists should further promote participation in management activities through peer-to-peer communication within the beekeeping community, by publishing papers on specialized magazines and advertising on Internet sites about beekeeping, decreasing their expenditures for communication campaigns on traditional media. Italy hosts approximately 40,000 amateur and 18,000 professional beekeepers, and their engagement in rapid detection and early warning activities could be fundamental to monitor the species at the national scale.

The seasonality of Internet searches about *V. velutina* seems to be correlated with the phenology of the species, since online searching volumes increased when people can observe the species in the environment (Mittermeier et al. 2019). Searches on Google increased during the activity season of the species, between April and October, with two peaks in May-June and August-September, corresponding to the first phase of nest construction and to the time of the year when colonies reach considerable size, becoming a concern for people. However, the observed seasonality could also be affected by the news published by the media or by ongoing conservation projects, which seems to have a higher frequency during the months of activity of the species (Lioy et al. 2019c).

Consultation to the Wikipedia page about the species was in the order of thousands of visits per month, with peaks of more than 10,000 visits. We believe that such a high number of visits is unlikely to have been generated by stakeholders or researchers alone, and that it probably involved laypeople as well. The overall volume of Internet searches about *V. velutina* and their long-term trend indicated a progressive awareness of laypeople about its progressive establishment into new areas and related consequences. This aspect was confirmed by the long term-trends of the Google Trends index for the two Italian words for the species, which increased between 2013 and 2020, reflecting the progressive spread of the species in Italy and the emergence of relevant impacts on beekeeping (Bertolino et al. 2016). The Google Trends index is discounted for the overall number of searches on Google, which strongly increased between 2013 and 2020 (Bologna et al. 2018): the fact that such index grew steadily during this period indicates that a growing proportion of people were interested in *V. velutina* and searched for it on the Internet.

The analysis of Internet searching volumes might be a promising complementary tool for monitoring the presence of *V. velutina* in Italy and Europe. As Google Trends can be downloaded at the regional level, peaks in searches about *V. velutina* could signal the species' colonization of a certain area. This approach is already adopted in epidemiology to trace the circulation of viral disease (Carneiro and Mylonakis 2009), and other studies considered it for the monitoring of common invasive alien species, such as the tiger mosquito (*Aedes albopictus*, Cerri and Bertolino 2020). As *V. velutina* actively exploits human buildings for constructing its nests, becoming visible and concerning to residents, this approach should work well in its invaded range due to the high proportion of urban and rural landscapes. Nevertheless, due to the high misidentification rate of the species with native insects, peaks in searches should be followed by insights or direct monitoring with traps (Demichelis et al. 2014; Lioy et al. 2020) for assessing with certainty the presence of *V. velutina*. Moreover, the analysis of seasonal patterns in relation to searches on Google and Wikipedia might also highlight spatial patterns in the phenology of the species in its invaded range, contributing to improve our understanding of how the phenological plasticity of invasive alien species affects their invasion success at a time of climate change (Manfredini et al. 2019).

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Supplementary material 1

Complete copy of the questionnaire on *Vespa velutina* in English and Italian language

Authors: Jacopo Cerri, Simone Lioy, Marco Porporato, Sandro Bertolino

Data type: PDF document

Explanation note: Complete copy of the questionnaire on *Vespa velutina* in English and Italian language.

Link: https://doi.org/10.3897/neobiota.73.80359.suppl1

Supplementary material 2

Distribution of scores to the questions on the perceived impacts of *V. velutina* and its severity in relation to various threats for beekeeping

Authors: Jacopo Cerri, Simone Lioy, Marco Porporato, Sandro Bertolino

Data type: PDF document

Explanation note: Distribution of scores to the questions on the perceived impacts of *V. velutina* and its severity in relation to various threats for beekeeping.

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RESEARCH ARTICLE

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Plankton hitch-hikers on naturalists' instruments as silent intruders of aquatic ecosystems: current risks and possible prevention

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Abstract

Organism dispersal is nowadays highly driven by human vectors. This also refers to the aquatic organisms that can often silently spread in and invade new waters, especially when human vectors of dispersal act without brakes. Thus, it is mandatory to continuously identify human-mediated mechanisms of organism dispersal and implement proper biosecurity treatments. In this study, we demonstrate how the plankton net – one of the basic instruments in the equipment of every plankton sampling person is a good vector for plankton dispersal and invasions. We also demonstrate whether keeping the net in an ethanol solution after sampling is a proper biosecurity treatment, and what kind of treatments are implemented by people worldwide. The first simulation shows that bloom-forming cyanobacteria can easily infiltrate into the new environment thanks to the nets, and can prosper there. However, ethanol-based biosecurity treatment efficiently prevented their spread and proliferation in the new environment. The second simulation, based on wild plankton from an eutrophic lake, indicates that a plethora of phyto- and zooplankton taxa can infiltrate into the new waterbody through the net and sustain themselves there if the net is only flushed in the waterbody and left to dry after sampling (an approach that is commonly used by naturalists). Here, we also show that native plankton residents strongly shape the fate of hitchhikers, but some of them like cyanobacteria can successfully compete with residents. Survey data alert us to the fact that the vast majority of biologists use either ineffective or questionable biosecurity treatments and only less than a tenth of samplers implement treatments based on disinfectant liquids. Our results emphasize that the lack of proper biosecurity methods implemented by the biologists facilitates the spread and invasions of plankton including also invasive species of a great nuisance to native ecosystems. Considering that naturalists usually use different instruments that might also be good vectors of plankton dispersal, it is necessary to develop proper uniform biosecurity treatments. No longer facilitating the plankton spread through hydrobiological instruments is the milestone that we, plankton samplers worldwide, should achieve together in the nearest future if we want to continue our desire to explore, understand, protect and save nature.

Keywords
Abiotic resistance, accidental species introduction, aquatic biomonitoring, biosecurity treatment, invasive species

Introduction

Dispersal is one of the main forces that shape the diversity of plankton communities. Numerous plankton species possess various morphological structures (hooks, threads, spines, suckers) and adaptations (buoyancy regulation, resting cells and eggs, gelatinous sheaths) that make them easy to be transported via different vectors for short and long distances. Thanks to dispersal, plankton organisms can invade new habitats, which may lead to establishing a stable population in a new waterbody. Thereby, plankton dispersal has an important influence on the biodiversity of aquatic ecosystems.

Plankton organisms can disperse outside of their existing distributions using different natural vectors: wind, extreme events, channels or rivers, other organisms such as birds, semi-aquatic and land animals (Havel and Shurin 2004; Bergström et al. 2008; Solarz et al. 2020). The dynamic change of the structure and composition of ecosystems has always occurred as an outcome of natural processes. However, nowadays organism dispersal is highly driven by increased human activity and human-made devices, instruments and actions (Havel and Shurin 2004). For example, Carlton and Geller (1993) were some of the first researchers to show that aquatic organisms can be easily transported across the oceanic barriers in ships' ballast water. Simulations performed by Hyun et al. (2016) revealed that some of the plankton travellers could survive the journey in ballast water and establish stable populations in the new environment. In some circumstances, e.g. other climatic conditions in relation to native conditions, silent invaders can express specific phenotypic adaptations (Miglietta and Lessios 2009). Nonindigenous plankton species can also get into new waters during the stocking of fish in the water bodies. Such a mode of introduction has been hypothesized for exotic cladoceran *Daphnia lumholtzi*, which appeared in some North American lakes at the beginning of the 1990s (Sorensen and Sterner 1992; Havel and

Hebert 1993) and currently is widely distributed in many different aquatic habitats in North America (Benson et al. 2022).

Because humans will continue to interact with aquatic environments, it is impossible to completely stop the human-aided dispersal of aquatic organisms. What we can do is minimise the risk of occurrence of human-mediated organism dispersal events by the implementation of biosecurity methods in human activity. For example, ballast water is subjected to different disinfection techniques before discharge (Hess-Erga et al. 2019), which consequently lowers the risk of spreading the ship's nonindigenous hitch-hikers in the new environment. However, we should be aware that many other kinds of activity in the aquatic environment that neglect a proper biosecurity method(s) might facilitate the spread of aquatic organisms. The problem increases if the species with a high invasive potential are present in the environment and on human-mediated vectors of an organism dispersal act without any biosecurity treatments. This may facilitate fast expansion of invasive organisms in a given region, which consequently hinders water bodies management, threatens the native species (Bøhn et al. 2008), can support a spread of new diseases (Martín-Torrijos et al. 2021), and generally leads to a deterioration in the ecosystem quality (Pinero-Rodríguez et al. 2020). Thus, continuous identification of other human-mediated mechanisms of plankton organism dispersal as well as development of proper biosecurity treatments are necessary.

In this study, we checked the possibility of the spread of lake plankton between different water bodies through a plankton net during sampling campaigns and whether disinfection of the plankton net with ethanol after sampling might be an efficient method to prevent the transfer of plankton net hitch-hikers into the next waterbody to be sampled. There were two experiments performed. In the first one, we tested whether plankton sampling with a net could facilitate the spread of some filamentous cyanobacteria and their potential invasion of new water bodies; we also tested if the use of biosecurity would prevent their spreading. Our attention was focused on cyanobacteria – organisms that often constitute a dominant component of plankton in different types of aquatic habitats and form dense blooms, generating an avalanche of problems to the functioning and usage of aquatic ecosystems (Waajen et al. 2014; Krztoń et al. 2019; Moustaka-Gouni and Sommer 2020). Many cyanobacteria are known producers of potent toxins with noxious effects on humans as well as on many aquatic and terrestrial organisms, which further highlights the need to control their spread. In the second experiment, we assessed the spreading success of plankton net hitch-hikers from an eutrophic lake in the filtrate from another lake containing or lacking a resident green alga *Tetradesmus obliquus*. In addition to the experimental part of the work, we performed a short survey among plankton samplers worldwide. In this way, we got an insight into what kind of biosecurity treatments they use during plankton sampling via nets, and what has been changed in the way of naturalists' actions during their work since the recalls of some scientists (Talling 1951; Padisák 2003; Padisák et al. 2016) about the problem of plankton dispersal through hydrobiological instruments.

Methods and materials

Dispersal of cyanobacteria via a plankton net, the fate of hitch-hikers, and possible prevention

We checked whether plankton sampling by a net with and without biosecurity could facilitate the spread of cyanobacteria and their invasion of new water bodies. Four laboratory strains of common bloom-forming filamentous cyanobacteria species such as *Aphanizomenon gracile*, *Aphanizomenon flos-aquae*, *Planktothrix agardhii*, and *Raphidiopsis raciborskii* (Table 1) were used for this test.

Table 1. Basic information on the origin (lake/reservoir name, GPS coordinates) of examined strains of cyanobacteria and their toxins (symbols +, –, n.e. indicate that a given toxin was detected, not detected or not examined, respectively).

Toxin	*A. flos-aquae*		*A. gracile*		*P. agardhii*		*R. raciborskii*	
	AMU-DH-6		**AMU-DH-1**		**SAG 6.89**		**SAG 1.97**	
	Sulejów Reservoir		**Lake Buszewskie**		**Lake Lough Neagh**		**Lake Balaton**	
	51°26'0"N, 19°55'25"E		**52°32'42"N, 16°22'47"E**		**54°11'0"N, 10°26'45"E**		**46°48'51"N, 17°45'52"E**	
Anabaenopeptins	–	(†)	+	(‡)	+	(†)	+	(‡)
Anatoxin-a	–	(†)	–	(‡)	–	(†)	–	(‡)
β-Methylamino-L-alanine	–	(†)	–	(‡)	–	(†)	–	(‡)
Cylindrospermopsin	–	(†)	–	(¥)	–	(¶)	–	(¶)
Desmethyl microcystin-LR	–	(†)	–	(¥)	+	(¶)	–	(¶)
Desmethyl microcystin-RR	–	(†)	–	(¥)	+	(¶)	–	(¶)
Microcystin-LR	–	(†)	–	(¥)	+	(§, ¶)	–	(¶)
Microcystin-RR	–	(†)	–	(¥)	+	(§, ¶)	–	(¶)
Microcystin-YR	–	(†)	–	(¥)	+	(§, ¶)	–	(¶)
Microcystin-LF	–	(†)	–	(¥)	–	(¶)	–	(¶)
Microcystin-LW	–	(†)	–	(¥)	–	(¶)	–	(¶)
Microcystin-LY	–	(†)	–	(¥)	–	(¶)	–	(¶)
Nodularin	–	(†)	–	(†)	–	(†)	*n.e.*	
Saxitoxins	–	(†)	–	(‡)	–	(†)	–	(‡)

Symbols: † this study is the first report; ‡ Falfushynska et al. (2021); § Kosiba et al. (2019); ¶ Wejnerowski et al. (2018); ¥ Wejnerowski et al. (2020). Detailed methodology and data of toxicological analyses performed for the purpose of this study are described in Suppl. material 1.

Twenty-day-old cyanobacterial cultures were inoculated into 70 L of distilled water. After 5 min. of gently mixing the volume of the container, we collected three subsamples of water for chlorophyll-*a* and microscopic analyses of cyanobacteria (respectively: 80 ml × 3, 1.5 ml × 3). Subsequently, 10 handmade plankton nets (mesh size: 100 μm; Suppl. material 2) were separately and fully immersed in water with cyanobacteria for 5 seconds. A large mesh size has been deliberately chosen to make the experiments more rigorous and leading to stronger conclusions (smaller mesh size of the net potentially means a higher risk of plankton retention on the net). The volume of the container was mixed for 30 seconds before each net was dipped. Net sample buckets were open during the procedure, which enabled an immediate outflow of water after pulling the net out of water. Five randomly selected nets were then left freely in empty plastic bags for 3 hours (treatment: Biosecurity(–)). This procedure simulated an approximate travel time from one sampling point to another and was estimated arbitrarily. The rest of the nets were entirely immersed for 3 hours in a 30% ethyl alcohol solution in distilled water (treatment: Biosecurity(+)) to see if it can effectively prevent the spread of cyanobacteria. After that, every net was separately immersed for 5 seconds in a beaker filled with 2.5 L of WC medium (Guillard and Lorenzen 1972). The beakers were then placed in a walk-in phytotron chamber (Conviron, Winnipeg, Canada) for seven days at a defined temperature (20 ± 0.5 °C), light intensity (40–50 µmol photons m^{-2} s^{-1}), and photoperiod (16:8 hours light:dark cycle). At the beginning and the end of the incubation, samples from each beaker were collected for chlorophyll-*a* (80 ml) and microscopic analyses (1.5 ml). Before sampling, the content of each beaker was gently mixed for 10 seconds.

Dispersal of the wild plankton via a net and the fate of hitch-hikers

Here, we checked whether plankton sampling with a net without biosecurity could facilitate the spread of lake phyto- and zooplankton and their invasions of new water bodies. For this purpose, a shallow postglacial open lake – Gopło (central Poland) was visited and sampled in October 2019. We chose this lake because of its high susceptibility to cyanobacterial blooms known from the past several decades (Burchardt and Podolski 1992). At the time of our sampling campaign, the lake was dominated by *Planktothrix agardhii*. Detailed characteristics of the Gopło Lake, including cyanotoxin presence, are outlined in Suppl. material 3.

At first, we collected three 300 ml subsamples of the lake water for a chlorophyll-*a* analysis and a 1 L sample for a microscopic analysis. Second, 15 plankton nets (the same nets as used in the first experiment) with open sample buckets were separately and fully immersed for 5 seconds in Gopło's waters at a depth of ~1.5 m. Subsequently, they were placed freely in plastic bags in a car Volkswagen Caddy III cargo (Volkswagen Group, Wolfsburg, Germany).

After 4.5 hours, the nets were returned to the laboratory. Five randomly selected nets were separately immersed for 5 seconds in beakers filled with 3 L of distilled water. After gently mixing, half of the volume was taken from each beaker and fixed with Lugol's solution for a qualitative/quantitative phyto- and zooplankton analysis. This procedure was to reveal Gopło's plankton net hitch-hikers, which infiltrated into a new waterbody. The remaining ten nets were separately immersed for 5 seconds in beakers filled with 3 L of filtrate (GF/C filters, Whatman, Maidstone, United Kingdom) water from another eutrophic lake – Kierskie (western Poland; GPS coordinates: 52°28'25.6"N, 16°46'59.7"E). Five of them were additionally inoculated with 30 ml of a green alga *Tetradesmus obliquus* (strain SAG 276-3a). This procedure was to see the fate of Gopło's potential plankton net hitch-hikers in a new waterbody when a resident is present. All beakers were maintained in a walk-in phytotron for ten days.

On day 0 and after ten days, beakers were sampled for a chlorophyll-*a* analysis (100 ml) and an assessment of *P. agardhii* biomass (1.5 ml). At the endpoint, we have also collected 50 mL samples for nutrient analyses and 1 L samples for a qualitative/quantitative analysis of the whole phyto- and zooplankton community structure. This procedure was to see the successes and failures of Gopło's potential plankton net hitch-hikers after ten days in the new water bodies.

Sample analyses

Chlorophyll-a concentration

Samples were filtered through GF/C filters using a glass vacuum filtration kit and analysed spectrophotometrically following grinding of the filters using a mortar and subsequent 24 hours extraction in 90% acetone (Wetzel and Likens 2000).

Cyanobacteria and green algae biomasses

Samples (1.5 ml) collected from the experiments were analysed using an inverted microscope Leica DM IL LED (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera Jenoptik ProgRes Speed Xtcore3 (Jenoptik Optical Systems, Jena, Germany). Trichomes of *A. gracile*, *P. agardhii*, and *R. raciborskii* from the first experiment and *P. agardhii* from the second experiment, were counted in a Sedgewick-Rafter chamber (Cole-Parmer, Vernon Hills, United States of America). Morphometry of cyanobacterial trichomes was assessed using ProgRes image capture software (Jenoptik Optical Systems, Jena, Germany). The thickness (n = 15) and length (n = 30) of randomly selected trichomes of each species were measured in each sample. Then, the average biovolume of each species was calculated using a formula for computing the volume geometric shape corresponding to the geometric shape of a given species (Vadrucci et al. 2007). Abundance and biovolume data were used to calculate the initial and final biomass of each species according to the following formula:

$$B(\mathrm{mm^3L^{-1}}) = \mathrm{Abundance} \times \mathrm{Biovolume}$$

Phyto- and zooplankton community composition

Samples (1 L) collected from Experiment 2 were subjected to sedimentation for a month, subsequently concentrated up to 50 ml and additionally fixed with Lugol's solution. The qualitative (the number of taxa and species composition) and quantitative (abundance expressed as the number of individuals of a given taxon/species L^{-1}) analyses of the Gopło's phytoplankton and Gopło's hitch-hikers were performed using an Olympus BX 51 microscope with Nomarski differential contrast (Olympus, Tokyo, Japan). Individuals (single cells, coenobia, colonies, filaments with a length of 100 μm) were counted in Fuchs-Rosenthal counting chamber (height: 0.2 mm, area: 0.0625 mm^2) at varied magnifications, i.e. microplankton in the whole chamber (512 fields) using 200× and nannoplankton in 256 fields using 400×. Phytoplankton identification was based on current references (e.g., Siemińska 1964; Starmach 1968; Ettl 1983; Ettl and Gärtner 1988; Hindák 1988; Starmach 1989; Popovský and Pfiester 1990; Komárek and Anagnostidis 1998, 2005; Wołowski and Hindák 2003; Komárek 2013; Lange-Bertalot et al. 2017).

The qualitative (the number of taxa and species composition) and quantitative (abundance expressed as the number of individuals of a given taxon/species L^{-1}) analyses of Gopło's zooplankton hitch-hikers were performed using a light microscope Axioskop 2 MOT+ (Carl Zeiss Light Microscopy, Oberkochen, Germany). Zooplankters of three groups (Cladocera, Copepoda, Rotifera) were determined and counted in a Sedgewick-Rafter chamber. If necessary, some animals were dissected after analysing the content of the whole chamber to be completely certain of the identification. Zooplankton identification followed Amoros (1984), Bielańska-Grajner et al. (2015), Błędzki and Rybak (2016).

Plankton sampling – survey

A short online survey was designed directly to gather information from plankton samplers worldwide about what they do with the plankton net after sampling and what biosecurity treatment they use. Questions in the survey were close-ended and addressed the following issues: frequency of plankton sampling with a net, the number of sampling points a day, the used methods of biosecurity after sampling, profession and degree status of the respondent (Suppl. material 4). In the case of some questions, participants had the opportunity to provide a short open-ended response to clarify or specify the response.

The survey was targeted at samplers who had an activity in the field of water research visible in the form of a scientific publication(s) indexed in the Web of Science citation database (Clarivate Analytics PLC, Philadelphia, United States of America) between the years 2000–2019. We used the keyword "plankton" in the WoS database search box to find proper participants for our survey. The scientists whose e-mails showed up in the database for the above search range were invited to respond to the survey (10,306 potential respondents). The survey was conducted in English and hosted on the SurveyLab online platform (www.surveylab.com; 7 Points Sp. z o.o., Warsaw, Poland). Each participant could complete the survey only once between March 2 and March 15, 2020.

The survey was voluntary and anonymous. Due to the hosting platform, we avoided collecting sensitive data from respondents. Also, we were unable to identify the respondents and link them to their answers. The exception was when a respondent sent an e-mail directly to the handling author of the survey with additional comments, suggestions, and the desire for a broader discussion.

In the analysis of the survey data, we did not include respondents sampling the marine environment. We also excluded from the analysis those respondents that answered to Q3 ("How many water bodies (e.g. lakes, reservoirs, ponds) do/did you sample for plankton using plankton net per day?") that they sample/sampled always one water body per day while later (Q5: "Do/Did you use different nets for each sampled lake when you sample/sampled more than one on the same day?") they did not mark the answer "I do/did not sample more than one lake on the same day". For the same reason, we excluded from the analysis those respondents that marked another answer than "Always one per day" to Q3 while later to Q5, they marked "I do/did not sample more than one lake on the same day".

Statistical analyses

Statistical analyses and graphs were conducted using the R software version 4.1.2 (R Core Team 2021), RStudio version 2021.9.2.382 (R Studio Team 2022). A Welch's paired t-test was applied to test the difference between chlorophyll-*a* concentration, the biomass of particular cyanobacteria strains at the start- and endpoint of the cultivation in Biosecurity(–) treatment (Experiment 1). Here, we used *rstatix* package (Kassambara 2020). Test assumptions were checked visually (Q-Q plots) and tested with formal methods (normality checked by the Shapiro–Wilk's test for the differences). The chlorophyll-*a* data in Biosecurity(–) contained one extreme outlier. Square-root transformation eliminated the problem and enabled us to run a classically paired t-test. Two outliers were detected in *P. agardhii* biomass data of Biosecurity(–) treatment, and data transformation did not solve it. Thus, *P. agardhii* biomass between two points of the cultivation was compared using Yuen's trimmed means test for two dependent samples using the *yuend* function in the *WRS2* package (Mair and Wilcox 2020). Data obtained from the Biosecurity(+) treatment were self-evident, and there was no necessity to seek a statistical confirmation.

In the case of experiment 2, Yuen's trimmed means test for two dependent samples was applied to test the difference in chlorophyll-*a* concentration between the start- and endpoint of the incubation of Gopło's hitch-hikers without a resident (Resident(–)). This test was used because these data contained one outlier, and data transformation did not solve the problem. The difference in chlorophyll-*a* concentration between the start- and endpoint of the incubation of Gopło's hitch-hikers with a resident *T. obliquus* (Resident(+)) was tested using Welch's paired t-test. The difference between Resident(–) and Resident(+) treatments in the biomass yield of Gopło's hitch-hiker *P. agardhii* at the endpoint of the incubation was tested using Yuen's trimmed means test. It was due to the presence of one outlier in the Resident(–) treatment.

Data were visualized using *GGally* (Schloerke et al. 2021), *ggplot2* (Wickham 2016), and *gridExtra* (Auguie 2017) package.

Results

Dispersal of cyanobacteria via a plankton net, the fate of hitch-hikers, and possible prevention

Cyanobacteria successfully spread into the new environment and withstood there when no biosecurity was applied (Biosecurity(–)), as positive values of the chlorophyll-*a* concentration and biomass of each species of cyanobacteria were detected immediately after the inoculation (Fig. 1). The chlorophyll-*a* concentration in beakers of the Biosecurity(–) treatment additionally increased by the end of the incubation (t = -13.4, DF = 4, p = .0001) (Fig. 1). The biomass of *A. gracile* increased almost threefold within the seven days after the inoculation (t = -4.07, DF = 4, p = .01), while the biomass of *P. agardhii* and *R. raciborskii* was stable (respectively: t = 1.31, df = 2, p = .32; t = 1.36, DF = 4, p = .24) (Fig. 1). *A. flos-aquae* was excluded from the analysis of biomass because this strain permanently formed fascicles visible in the beakers to the naked eye (Suppl. material 5). Some fascicles reached a length of ~2 cm.

In the Biosecurity(+) treatment, chlorophyll-*a* and cyanobacterial trichomes of each species were not detected in samples at both sampling points.

Dispersal of the wild plankton via a net and the fate of hitch-hikers

Chlorophyll-a concentration and phytoplankton

Chlorophyll-*a* concentration in the Gopło Lake was 79.1 ± 6.89 µg L^{-1} (mean ± SD). Wild phytoplankton successfully infiltrated the new environment and withstood there regardless of the presence or absence of the resident alga *T. obliquus*. Chlorophyll-*a* concentration in beakers without the resident *T. obliquus* increased over five times during the incubation time (t_y = -4.2, DF = 2, p = .052) (Fig. 2). In the case of beakers containing resident *T. obliquus*, the concentration of chlorophyll-*a* did not markedly differ between the start- and endpoint of the incubation (t = 1.44, DF = 4, p = .22).

Gopło's phytoplankton and hitch-hikers

Phytoplankton in the Gopło lake was represented by eight groups, including Chlorophyta (44 taxa), Bacillariophyceae (31 taxa), Cyanobacteria (10 taxa), Cryptophyceae (7 taxa), Euglenophyceae (5 taxa), Dinophyceae (3 taxa), Chrysophyceae (1 taxon), and Haptophyceae (1 taxon) (Fig. 3). Cyanobacterium *P. agardhii* was a dominant species, which accounted for 63% of the total phytoplankton density in Gopło.

Individuals of thirty-three phytoplankton taxa infiltrated the beakers with the Kierskie lake water via plankton nets (Fig. 3). Their identification resulted in the detection of Bacillariophyceae (13 taxa), Chlorophyta (11 taxa), Cyanobacteria (7 taxa), Cryptophyceae (1 taxon), and Euglenophyceae (1 taxon). Among all hitch-hikers,

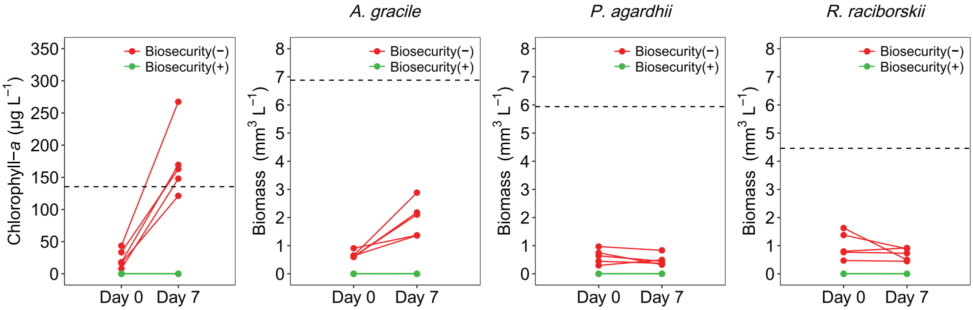

Figure 1. Chlorophyll-*a* concentration and biomass of *A. gracile*, *P. agardhii* and *R. raciborskii* at the start and endpoint of the incubation in Biosecurity(–) and Biosecurity(+) treatments. A dashed line indicates the mean value of a given variable in the container with a simulated bloom of cyanobacteria in which plankton nets were immersed before the experiment.

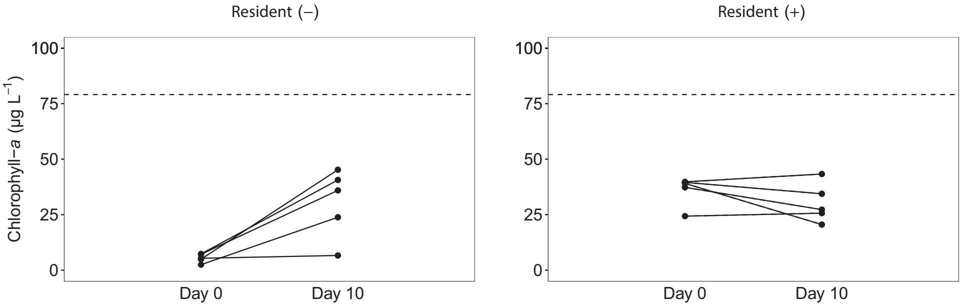

Figure 2. Chlorophyll-*a* concentration in beakers with Gopło's plankton net hitch-hikers at the beginning and the end of the incubation without or with resident *T. obliquus*. Horizontal line indicates chlorophyll-*a* concentration in the Gopło Lake.

P. agardhii was the most numerous in the beakers: it constituted on average 89% of the total phytoplankton density.

After ten days of hitch-hikers' incubation without resident *T. obliquus* (Resident(–)), thirty-seven phytoplankton taxa were found in beakers (Fig. 3): Chlorophyta (20 taxa), Bacillariophyceae (10 taxa), Cyanobacteria (5 taxa), Cryptophyceae (1 taxon), Chrysophyceae (1 taxon). *P. agardhii* was the dominant species in these beakers. Its density ranged from .42 to 5.78 million individuals L^{-1} and constituted on average 66% of the total phytoplankton density. Chlorophyte *Mougeotia* sp. was the second most abundant taxon: its density constituted almost 12% of the total phytoplankton density.

After ten days of hitch-hikers' incubation with resident *T. obliquus* (Resident(+)), fourteen phytoplankton taxa were found in beakers (Fig. 3): Bacillariophyceae (6 taxa), Chlorophyceae (4 taxa), Cyanobacteria (3 taxa), and Euglenophyceae (1 taxon). Chlorophyte *T. obliquus* (the resident strain plus conspecifics transferred from the Gopło lake) was the dominant species in these beakers: its density ranged from 5.5 to

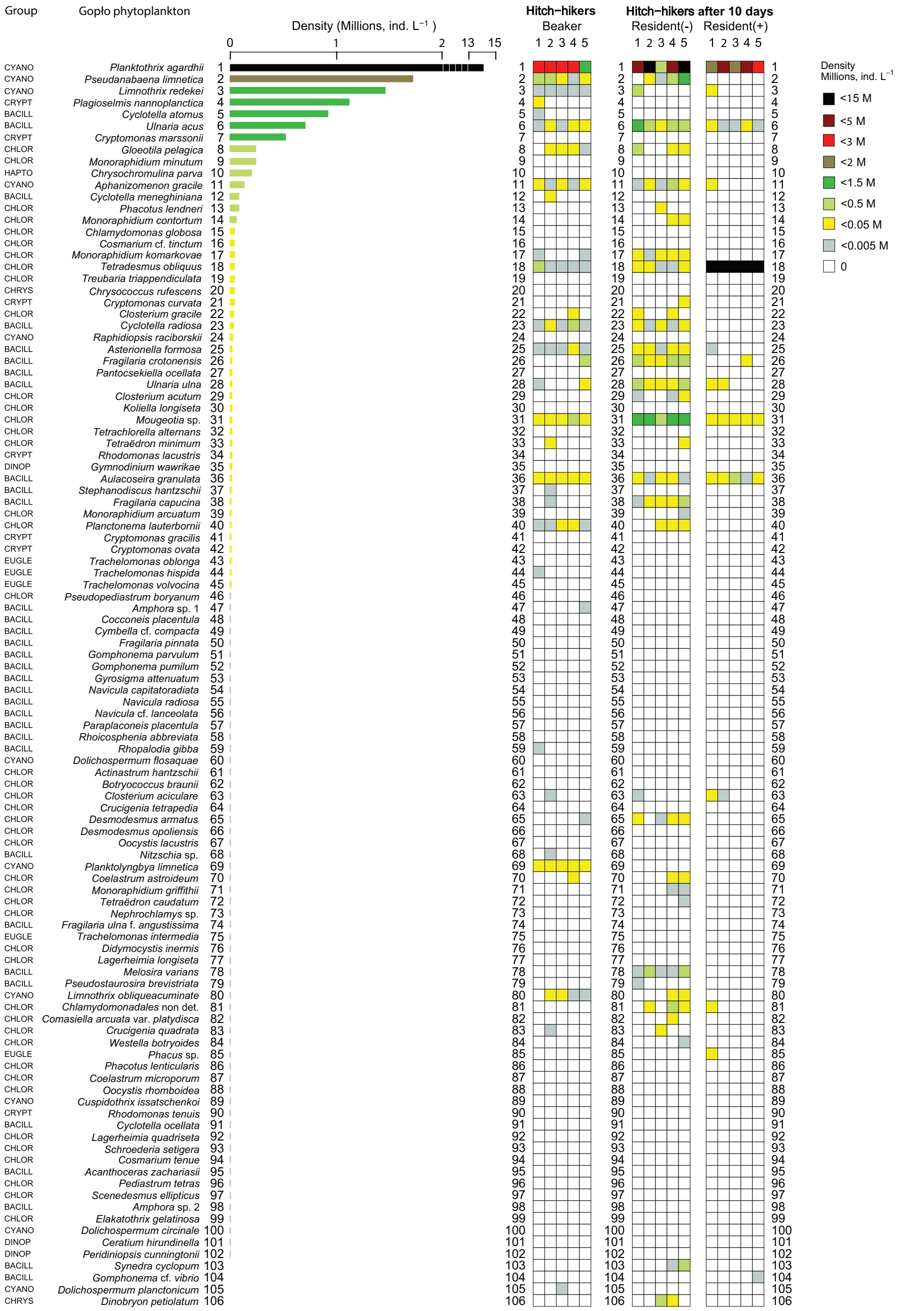

Figure 3. Phytoplankton community structure in the Gopło lake, phytoplankton hitch-hikers infiltrated into beakers through plankton nets and their fate after ten days of incubation in filtered lake water from the Kierskie lake without and with resident *T. obliquus* (Resident(–), Resident(+); respectively). Abbreviations: CYANO – Cyanobacteria, CRYPT – Cryptophyceae, BACILL – Bacillariophyceae, CHLOR – Chlorophyta, DINOP – Dinophyceae, HAPTO – Haptophyceae, EUGLE – Euglenophyceae, CHRYS – Chrysophyceae.

10.6 million individuals L^{-1} and constituted 73% of the total phytoplankton density. *P. agardhii* was the second most abundant taxon in the beakers: its density ranged from 1.68 to 4.08 million individuals L^{-1} (26% on average of the total phytoplankton density).

Effect of resident on Gopło's dominant hitch-hiker

At the start of the incubation, *P. agardhii* biomass in Resident(–) and Resident(+) was, respectively, .99 ± .16 and 1.09 ± .19 $mm^3\ L^{-1}$ (mean ± SD). After 10 days of incubation, *P. agardhii* biomass in the Resident(–) treatment increased up to 7.42 ± 3.55 $mm^3\ L^{-1}$ and was over a half higher than in the Resident(+) treatment (3.15 ± 1.8 $mm^3\ L^{-1}$) ($t_y = 4.14$, DF = 8, $p = .01$) (Fig. 4).

Gopło's zooplankton and hitch-hikers

Zooplankton community in Gopło was represented mainly by rotifers (Fig. 5). Rotifer *Keratella cochlearis* was a dominant species, which comprised 69% of total Gopło's zooplankton density. There were two forms of *K. cochlearis*, f. typica and tecta, and the density of both forms ranged between 200–300 individuals L^{-1} with spineless f. tecta being slightly more numerous. The density of other taxa was lower than 100 individuals L^{-1}. The total density of zooplankton in the Gopło Lake was 718 individuals L^{-1}.

Three Rotifer taxa, four Cladocera taxa, one Copepoda taxon, and larval and juvenile forms of Copepoda (Calanoida and Cyclopoida) infiltrated the beakers with the lake water through plankton nets in quantities large enough to be detected upon first sampling (Fig. 5). *Keratella quadrata*, Cyclopoida nauplii and *Keratella cochlearis* f. typica were the most abundant in the beakers and constituted, respectively, 41.99%, 29.51%, and 12.5% of the total zooplankton density. The total density of zooplankton was on average 217.6 ± 110.6 individuals L^{-1}. After ten days of Gopło's hitch-hikers' incubation without resident *T. obliquus* (Resident(–)), six zooplankton taxa were found in beakers: Rotifera (4 taxa), Cladocera (2 taxa). Nauplii and copepodites of Cyclopoida and copepodites of Calanoida copepods were also detected. The total density of zooplankton in Resident(–) was 731 ± 1289.5 individuals L^{-1}. Nine zooplankton taxa were found on day 10 in beakers with resident *T. obliquus* (Resident(+)): Rotifera (4 taxa), Cladocera (3 taxa), Copepoda (2 taxa). There were also nauplii and copepodites of Calanoida and Cyclopoida. Four Rotifer taxa, *K. quadrata*, *Lecane* sp., *K. cochlearis* f. typica, and *Brachionus* sp. were most abundant in the community, and they constituted, respectively, 46.59%, 26.27%, 17.28%, and 8.49% of the total zooplankton density. The total density of zooplankton in Resident(+) was 1378 ± 481.39 individuals L^{-1}.

Biosecurity measures

After data filtration (see "Plankton sampling – survey" subsection), responses from 388 respondents were used in the analysis of the survey data, including 340 (88%) broadly understood biologists at a university or freelancer biologists, 12 (3%) employees at

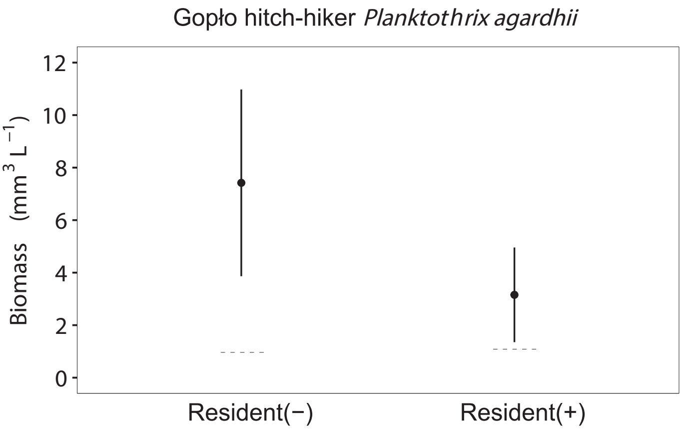

Figure 4. Average biomass (± SD) of Gopło's hitch-hiker *P. agardhii* at the endpoint of the incubation in the Resident(–) and Resident(+) treatments. Dashed horizontal lines indicate the average biomass of Gopło's hitch-hiker *P. agardhii* at the start point of the incubation.

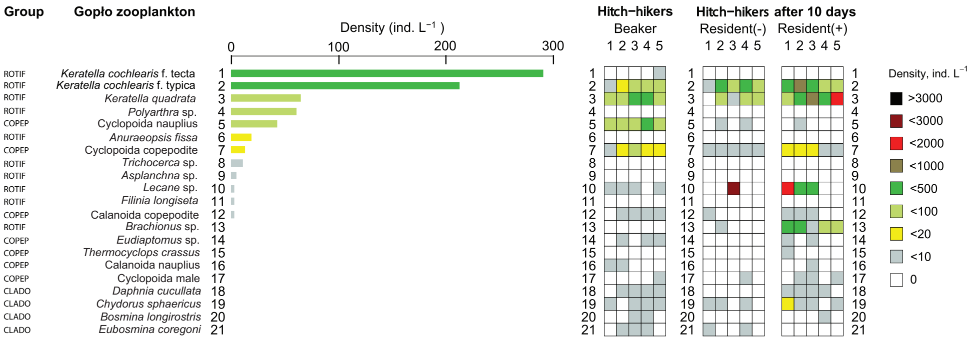

Figure 5. Zooplankton community structure in the Gopło lake, zooplankton hitch-hikers infiltrated into beakers through plankton nets and their fates after ten days of the incubation in filtered lake water from the Kierskie lake without and with resident *T. obliquus* (Resident(–), Resident(+); respectively). Abbreviations: ROTIF – Rotifera, COPEP – Copepoda, CLADO – Cladocera.

water quality monitoring service and 36 (9%) people of another profession. The responses came from 58 countries (Suppl. material 6). Our pool included professors (158 respondents, 41%), naturalists with a doctoral degree (184, 47%), PhD students (21, 5%), naturalists with a Master's degree (22, 6%) and a Bachelor's degree (3, 1%).

Regarding the frequency of plankton sampling, most respondents reported that they sample/have sampled plankton with a plankton net irregularly, including years without sampling (33%). Regular plankton sampling, either usually more than twenty times a year or usually up to ten times a year was reported, respectively, by 21% and 20% of respondents. Regular sampling, usually from eleven to twenty times a year, was reported by 14% of the respondents. Irregular sampling of up to ten times a year in some years and more often in other years was declared by 11% of the respondents. There were also 1% of respondents that marked the response "None of the above".

Regarding the number of water bodies sampled per day, 39% of respondents declared to sample usually one water body per day and rarely more than one, 38% of the respondents reported to sample usually more than one but not more than five water bodies per day, 17% indicated that they always sample one water body per day. Another 6% of respondents usually sample more than five water bodies per day.

Among those who sample more than one lake a day, only 22.5% of respondents declared to use a different plankton net for each lake, while 77.5% use the same net in all sampled lakes.

A little over a half of the respondents declared to always rinse/have rinsed an open plankton net after sampling with distilled water/tap water and let it dry (Fig. 6).

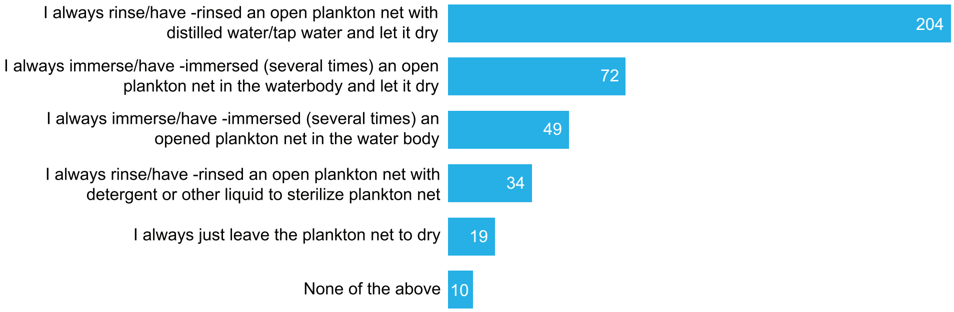

Figure 6. Biosecurity procedure(s) used by plankton samplers worldwide with the plankton net after sampling. Numbers inside the bars indicate the number of respondents who marked a given response.

Table 2. The detailed explanations that were provided by the 3% of respondents who chose the answer "None of the above" when asked what they do with plankton net after sampling.

No.	*Original answers*
1	"Rinse with distilled water if sampling in the same water body, wash with detergent if using in different water bodies."
2	"We use separate plankton nets for each lake - one lake, one plankton net. But we rinse it 10 times after sampling and 10 times before sampling next month."
3	"It depends, if there is a risk of spreading invasive species, I sterilise and then dry the net. Otherwise I take it back to the lab and rinse it with distilled water before letting it dry."
4	"After sampling I take the plankton net to the next sampling place and rinse there the net several times before sampling."
5	"Rinse and preserve in formalin."
6	"The sample should be concentrated and the net should be washed and dried."
7	"I don´t know it so precisely."
8	"Samples after draining are fixed with formalin or alcohol."
9	"I added formaldeyhde to a 4% concentration and buffer with hexamethiltetramine until a pH of 8.2."
10	"i."

The second most popular method used by 19% of the respondents is to always immerse (several times) an open plankton net in the water body and let it dry, while 13% of respondents admitted to always immerse (several times) an open plankton net in the water body. Only 9% of respondents indicated that they always rinse/have rinsed an open plankton net with a detergent or other liquid to disinfect the plankton net. There was also 5% of the respondents who always just leave the plankton net to dry, and 3% of the respondents that marked the "None of the above" response. These last 3% of the respondents were additionally asked to specify what they do with the plankton net after sampling and all the answers are outlined in Table 2.

Discussion

The results of our experiments demonstrate that a plankton net is an efficient vector for dispersion of plankton organisms. In the first experiment, all examined strains of cyanobacteria were able to disperse from the source into the new medium through the plankton net. They were able to survive three hours on the plankton nets out of the water and inhabit the new medium. There was one dominant species (*A. gracile*) that increased markedly in its biomass at the endpoint of the incubation; others (*P. agardhii*, *R. raciborskii*) maintained a relatively stable biomass during the experiment. There were also visible numerous enlarged *A. flos-aquae* fascicles at the endpoint of the incubation. All of these species often co-occur and compete for dominance in eutrophic waters of the temperate zone (Kobos et al. 2013). One of them, *R. raciborskii* has a tropical and subtropical origin and exhibits a great invasive potential (Antunes et al. 2015), and its presence in temperate waters was distinctly noticed in recent years (Wilk-Woźniak et al. 2016; Kokociński et al. 2017; Rzymski et al. 2018). It was predicted that cyanobacteria without a biosecurity treatment could be successfully spread by a plankton net and establish stable populations in the simulated new environment. After all, this experiment was conducted on laboratory strains and we used the same medium that the examined strains were customarily cultured in. Moreover, cyanobacterial trichomes had a high variability in length (since ~20 to ~1700 μm) which can make some of them easy to entangle and anchor in the nylon grid of the plankton net, even if the mesh size was 100 μm. The ability of cyanobacteria to secrete exopolysaccharides (EPS) that have adhesive and gluing properties (Cruz et al. 2020) might additionally help them to stick to the nylon threads of a net and temporarily protect cyanobacterial cells against desiccation as well (Costa et al. 2018). Although the ability of the examined strains to produce EPS has not yet been checked, literature data indicate that some members of the *Aphanizomenon*, *Planktothrix*, and *Raphidiopsis* genera possess this feature (respectively: Xue et al. 2015; Meccheri 2010; Zarantonello et al. 2018). Hence, a lack of proper biosecurity methods implemented during plankton sampling can facilitate spread of nuisance bloom-forming species and those of a high invasive potential. Toxicity of hitch-hikers seems to have no impact on their efficiency to spread between the water bodies through a plankton net. All of the tested strains had relatively stable populations at the endpoint of the incubation. *A. flos-aquae* was free of the most common cyanotoxins (anabaenopeptins, anatoxin-a, β-N-methylamino-L-alanine, cylindrospermopsin, microcystins, nodularin, saxitoxin), *A. gracile* and *R. raciborskii* produced anabaenopeptins only and *P. agardhii* produced anabaenopeptins and some microcystins.

Keeping the plankton net in 30% ethanol after sampling seems to be a promising method to prevent the spread of cyanobacteria between different water bodies via the plankton net. After seven days of incubation, neither chlorophyll-*a* nor cyanobacterial trichomes/fascicles were detected in the medium. This result additionally points to the superiority of the biosecurity method based on disinfection with ethanol over only flushing the net in the water body and letting it dry. The biosecurity method using ethanol does not require a lot of work, time, and space in the vehicle, and is also relatively cheap (it only takes a bucket with a lid + ethanol + distilled/tap water). There are also other possible disinfectant liquids that could be used, but we believed that due to rapid evaporation, ethanol would have negligible negative effects on the environment. The advantage of biosecurity methods based on disinfectant liquids is that their power can be maximized by using a higher concentration of a disinfectant to get an assurance that other planktonic organisms and their propagules will also become irreversibly inactive when transferred into a new water body.

Identifying which phyto- and zooplankton species are effective or ineffective plankton net hitch-hikers was enabled in the second experiment, which was based on plankton from the Gopło Lake. Out of one-hundred and two Gopło's phytoplankton taxa, more than thirty of them were transferred into beakers with filtered lake water from another lake and survived ten days after the inoculation. A few zooplankton taxa from the Gopło Lake turned out to be successful intruders as well, some of them infiltrated the new simulated water body quite abundantly. There were numerous phytoplankton and only a couple of zooplankton hitch-hikers that expanded their abundance in the lake filtrate within ten days. The higher number of phytoplankton taxa found at the end point of the incubation in comparison to the start means that "no one is excluded from the game" and even those hitch-hikers that were initially easy to overlook due to low abundance can quickly proliferate in the new environment and succeed there. However, organisms with spines, bristles, and other protruding elements can have a greater chance of hitchhiking. For example, rotifer *K. cochlearis* f. typica with a caudal spine infiltrated the water more effectively than *K. cochlearis* f. tecta without the spine. This raises the question as to whether such spines provide a better chance of spreading on some of the natural vectors like bird feathers. The dominance of *P. agardhii* in the Gopło Lake and in the filtrates of the Kierskie Lake at the start- and endpoint of the experiment confirm that bloom-forming cyanobacteria can be easily spread by plankton samplers if there are no efficient biosecurity methods used. Because eutrophic waters are subjected to more frequent biomonitoring (due to e.g. phytoplankton bloom events, ongoing ecosystem restoration) and they often offer good conditions for the growth of nuisance plankton, efficient net hitch-hikers might have consequently a greater chance to spread and expand their distribution range. This might explain to some extent why numerous nuisance plankton taxa are cosmo- or at least subcosmopolitan. In the light of the 1) paradigm "everything is everywhere, but, the environment selects" (Baas-Becking 1934), 2) the significance of environmental heterogeneity for organismal distribution ("everything is not everywhere", Ribeiro et al. 2018), and 3) the increasing number and importance of human vectors and human-mediated natural vectors of organism dispersal, it seems that nowadays vectors are so powerful that they mainly decide where everything is, but the environment still selects and decides when everything has its own five minutes.

Predicting the fate of hitch-hikers is a great challenge as it depends on the resultant force of environmental factors in the new environment which these organisms and/or their propagules have to face. Among the factors determining winners and losers among newcomers, competitive abilities of native residents and biotic resistance of the whole native communities are often one of the most crucial (Dzialowski et al. 2007; Weithoff et al. 2017; Buchberger and Stockenreiter 2018). Our second experiment was too simple to get a deep insight into this problem; after all, in the treatment with "Resident(+)", we examined the effect of only one resident (*T. obliquus*) on the fate of plankton net hitch-hiker horde. Nevertheless, even the presence of one resident species strongly shaped the success of the hitch-hikers. The presence of laboratory strain of *T. obliquus* exacerbated the competition between all phytoplankters in the new environment. Consequently, the number of phytoplankton-hitch-hiker taxa at the endpoint of the incubation with resident *T. obliquus* was markedly reduced. Moreover, their abundance was generally lower than in the Resident(–) treatment and this referred as well to the dominant Gopło hitch-hiker *P. agardhii*. Some previous laboratory experiments (Mur et al. 1978; Ji et al. 2017) revealed that green algae are often strong competitors of bloom-forming cyanobacteria when there are no limiting factors for them, while our second experiment lasted only ten days and e.g. resources were generally not exhausted at the end point of the incubation (Suppl. material 7). Completely unlike phytoplankton, zooplankton hitch-hikers took advantage of the presence of resident *T. obliquus* which was manifested in the increased number of zooplankton taxa at the endpoint of the incubation. Moreover, some rotifer taxa increased in their biomass in comparison to the Resident(–) treatment. The high nutritious value and easy manageability of green algal cells or colonies in comparison e.g. to cyanobacteria (Bednarska et al. 2014; Sikora et al. 2014) possibly explain why the presence of the resident triggered positive effects on zooplankton newcomers. Moreover, zooplankton-hitch-hikers were composed of small-bodied taxa, mainly rotifers that can easily avoid less edible phytoplankton and its negative effects (Ger et al. 2016).

Getting insight into how aquatic ecologists worldwide prevent nowadays the spread of the plankton via the sampling net during their field works was also our "must-know" in this study. As revealed by the survey, less than a tenth of plankton samplers implement a biosecurity treatment based on disinfectant liquids (rinsing the net after sampling with detergent or other disinfectant liquid). The overwhelming majority of the respondents use other ways: 1) rinsing the net with open buckets after sampling with distilled/tap water, 2) immersing the net several times in the water body and letting it dry, 3) immersing the net in the waterbody without taking care about its dryness or 4) just leaving the net to dry. Based on the results of our two experiments, we already know that biosecurity treatment relying on flushing the net with an open bucket after sampling in the waterbody and letting it dry does not prevent plankton spread between the water bodies. This especially refers to the situation when sampling of several water bodies a day is planned, and considering the answers of our respondents on – "How many water bodies (e.g. lakes, reservoirs, ponds) biologists sample using plankton net per day?" – this practice is quite common in multiple sampling cases. Flushing the net after sampling in the water body and letting it dry fails as a biosecurity method also because it does not prevent the spread of plankton propagules (cysts, resting eggs and cells, spores etc.) that can well handle desiccation (Hori et al. 2003; Radzikowski 2013). This fact also puts the effectiveness of rinsing the net after sampling with distilled/tap water into question. Thus, it appears that, worldwide, the most commonly used biosecurity methods to prevent plankton spread between water bodies via the net are simply ineffective, and that the silent spreading and possible invasion can easily happen as a result of sampling. Our study has shown only a part of the problem. After all, aquatic naturalists use a variety of instruments and other necessary equipment during their work, and all these items, without proper biosecurity treatments, might also potentially be a vector for plankton spread between the water bodies. Moreover, we revealed this problem based on phyto- and zooplankton as a model. Whereas, considering that plankton organisms have their own "hitch-hikers" (bacteria, parasites, viruses; Grossart et al. 2010; Frada et al. 2014; Bass et al. 2021), it is likely that a lack of biosecurity treatments implemented by the naturalists might also facilitate the spread of pathogenic bacteria, parasitic and viral infections in the plankton communities between water bodies.

Conclusion

In summary, this study demonstrates that plankton net is an efficient vector for dispersion of plankton organisms. The fate of plankton net hitch-hikers in the new environment is strongly shaped by the native residents. A promising biosecurity method preventing the spread of plankton between water bodies is disinfection of the plankton net with an ethanol solution after sampling. Survey data indicate that the vast majority of people use either ineffective or questionable biosecurity treatments. No longer facilitating the plankton spread is the milestone that we, naturalists worldwide, should achieve together in the nearest future.

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CRediT authorship contribution statement: Wejnerowski Ł: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration. Aykut TO: Methodology, Investigation, Writing - Review & Editing. Pełechata A: Methodology, Software, Investigation, Writing - Review & Editing. Rybak M: Methodology, Software, Investigation, Writing – Review & Editing. Dulić T: Methodology, Investigation, Writing - Review & Editing. Meriluoto J: Methodology, Software, Investigation, Writing - Review & Editing. Dziuba MK: Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing.

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Supplementary material 1

Detailed methodology of toxicological analyses performed for the purpose of this study and the results in the graphical form

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: text + figures

Explanation note: Detailed methodology of toxicological analyses performed for the purpose of this study and the results in the graphical form. Abbreviations: APs – anabaenopeptins, ATX-a – anatoxin-a, BMAA – β-N-methylamino-L-alanine, CYN – cylindrospermopsin, MCs –microcystins, NOD – nodularin, STXs – saxitoxins.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl1

Supplementary material 2

Scheme of the handmade plankton nets used in the experiments and dimensions

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: figure

Explanation note: Scheme of the handmade plankton nets used in the experiments and dimensions.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl2

Supplementary material 3

Basic information about the Gopło Lake and some characteristics of the surface water measured during field campaigns

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: Table + text + figures

Explanation note: Basic information about the Gopło Lake and some characteristics of the surface water measured during field campaigns.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl3

Supplementary material 4

The list of questions and possible responses in the survey

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: text

Explanation note: The list of questions and possible responses in the survey.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl4

Supplementary material 5

Top view of the content of two randomly selected beakers of Biosecurity(–) treatment after seven days of incubation in a phytotron

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: image

Explanation note: Top view of the content of two randomly selected beakers of Biosecurity(–) treatment after seven days of incubation in a phytotron.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl5

Supplementary material 6

Survey responses by country

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: figure

Explanation note: Survey responses by country.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl6

Supplementary material 7

Concentration of ammonium, nitrate, orthophosphates in the Kierskie Lake filtrates (Resident(-), Resident(+)) at endpoint of the second experiment

Authors: Łukasz Wejnerowski, Tümer Orhun Aykut, Aleksandra Pełechata, Michał Rybak, Tamara Dulić, Jussi Meriluoto, Marcin Krzysztof Dziuba

Data type: figure

Explanation note: Concentration of ammonium (NH4+), nitrate (NO3-), orthophosphates (PO43-) in the Kierskie lake filtrates (Resident(-), Resident(+)) at endpoint of the second experiment.

Link: https://doi.org/10.3897/neobiota.73.82636.suppl7