

Limitations of invasive snake control tools in the context of a new invasion on an island with abundant prey

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Abstract

In October 2020, a new population of invasive brown treesnakes (*Boiga irregularis*) was discovered on the 33-ha Cocos Island, 2.5 km off the south coast of Guam. Cocos Island is a unique conservation resource, providing refuge for many lizards and birds, including endangered species, which were extirpated from mainland Guam by invasive predators including brown treesnakes. We sought to evaluate the usefulness of toxic baiting with acetaminophen-treated carrion baits and cage trapping, common tools for the control of brown treesnakes on mainland Guam, as potential eradication tools on Cocos Island. We evaluated multiple bait types and bait presentations: on the ground, suspended in the canopy emulating aerial bait applications and in four plastic-tube bait station configurations intended to exclude non-target species. We monitored all baits with time-lapse cameras. Despite improved exclusion of non-targets by bait station design, most baits were quickly removed by non-target species, particularly coconut crabs (*Birgus latro*) and Mariana monitors (*Varanus tsukamotoi*). Monitoring of 1,250 baits available for 2,427 bait nights resulted in no observations of brown treesnakes taking any bait. Subsequently, we tested two trap types commonly used on Guam and compared trapping success with live versus dead mouse lures. In 10,553 trap nights using live and dead mouse lures, we only captured one brown treesnake, in a trap with a live mouse

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lure. These baiting and trapping rates are so low as to be ineffectual for all practical purposes. Concurrent visual searching and hand capture of brown treesnakes during initial rapid response efforts demonstrates that these low baiting and trapping success rates are not a result of low snake density. We make a case for our assumption that the ineffectiveness of these tools on Cocos Island is due to the context of extremely high abundance of preferred live prey, primarily large geckos and birds. Our results have profound conservation ramifications, because any future island invasions by brown treesnakes are likely to occur within similarly prey-rich environments where these baiting and trapping methods might be similarly ineffective.

Keywords

bait stations, *Boiga irregularis*, camera traps, conservation, eradication feasibility, incipient population, non-target species, trapping

Introduction

The pace and scale of the introduction and spread of non-native reptiles continues to increase, as does recognition of the attendant ecological and economic harms they cause (Kraus 2009; Reed and Kraus 2010; Kraus 2015; Capinha et al. 2017). Snakes comprise a considerable proportion of these reptile invasions, many of which occur on islands where already-imperilled native species are at risk of extirpation or extinction by snake predation; examples include wolf snakes (*Lycodon aulicus*) on Mauritius and Réunion Island (Deso and Probst 2007), corn snakes (*Pantherophis guttatus*) and boa constrictors (*Boa constrictor*) on multiple Caribbean islands, California kingsnakes (*Lampropeltis californiae*) in the Canary Islands and multiple colubrids in the Balearic Islands (Tonge 1990; Perry et al. 2003; Bushar et al. 2015; Monzón-Argüello et al. 2015; Silva-Rocha et al. 2015). Invasive snake problems can be particularly intractable because of snakes' cryptic nature and ability to withstand long periods without feeding (Durso et al. 2011; Siers et al. 2018a; Yackel Adams et al. 2018; Boback et al. 2020; Nafus et al. 2020). To date, there are no known examples of eradication of an invasive snake population at a scale larger than 1 ha (Campbell et al. 2012; DIISE 2023).

The most well-known and well-studied example of an island snake invasion is that of the brown treesnake (*Boiga irregularis*) on the island of Guam in the Western Pacific. Accidentally transported from the Admiralty Islands to Guam in shipments of military equipment following World War II (Rodda and Savidge 2007; Richmond et al. 2015), this slender, nocturnal, arboreal predator spread throughout the entire island by the mid-1980s (Savidge 1987) and achieved densities unprecedented for any natural non-aggregating snake population (Rodda et al. 1999a). The spread of brown treesnakes across Guam was followed by a wave of negative impacts to all native vertebrate taxa (Wiles 1987; Fritts and Rodda 1998) including collapses in nearly all native bird populations, resulting in the extirpation or extinction of 12 of 15 native forest birds and the functional extinction of the island's entire forest avifauna (Savidge 1987; Wiles et al. 2003). This bird loss was followed by cascading effects on plants, invertebrates and ecological processes (Perry and Morton 1999; Rogers et al. 2012;

Caves et al. 2013; Fricke et al. 2014; Freedman et al. 2018). Socioeconomic damages caused by the brown treesnake invasion of Guam include ‘home invasions’ and painful bites to humans including infants (Fritts 1988; Fritts et al. 1990, 1994), predation on domestic animals including the loss of small-scale poultry production (Fritts and McCoid 1991; Rodda and Savidge 2007), declines in tourism (Hall 1996; Shwiff et al. 2010) and costs of power outages caused by snakes short-circuiting transmission lines (Fritts 2002).

Methods and strategies for brown treesnake control are being developed, tested and implemented for the protection and restoration of Guam’s native flora and fauna (e.g. Aguon et al. (2002); Siers et al. (2017a, 2020a, b); Clark et al. (2018); Klug et al. (2021a, b); Pollock et al. (2021)). However, the first and highest priority for invasive brown treesnake control has been interdiction—the prevention of further spread of this harmful predator from Guam to other vulnerable locations throughout the Pacific (Hall 1996; Stanford and Rodda 2007; Perry and Vice 2009; Clark et al. 2018; Engeman et al. 2018). The Commonwealth of the Northern Mariana Islands (CNMI, especially Saipan, Rota and Tinian) and the Hawaiian Archipelago are at particularly high risk of invasion and severe ecological and economic consequences (Fritts 1988; Shwiff et al. 2010; BTSTWG 2015; Yackel Adams et al. 2021). Before the implementation of a full interdiction programme on Guam, live brown treesnakes were too-commonly found in cargo from Guam to Saipan, Hawaii and other destinations; since a USDA Wildlife Services operational control programme began in 1993, such encounters have dropped to nearly zero and Saipan continues to be considered snake-free (Hall 1996; Stanford and Rodda 2007; Yackel Adams et al. 2018, 2021).

Cocos Island (CHamoru name: *Islan Dãno*) is a small atoll island situated approximately 2.5 km off the southern tip of the main island of Guam. Cocos Island was considered to comprise the majority of remaining snake-free habitat in Guam and is home to many vertebrates susceptible to brown treesnake predation, including some species that no longer persist on mainland Guam. Guam rails (*Hypotaenidia owstoni*: ko’ko’), once extinct in the wild due to brown treesnake predation, were introduced to Cocos Island where they have reproduced and thrived (Medina and Aguon 2000). The endangered Mariana skink (*Emoia slevini*) was extirpated from mainland Guam by brown treesnake predation, but a remnant population was recently rediscovered on Cocos Island (USFWS 2019). Other species, including regionally endemic lizards and birds, also persist on Cocos Island (Rodda and Fritts 1992).

While the high volume of commercial and military cargo and vessels originating from central and northern Guam has been scrupulously inspected for stowaway snakes, traffic between southern Guam and Cocos Island has received relatively little attention. A biosecurity plan was developed for the Island (USDA Wildlife Services 2009) to monitor for incursions of cats, rodents and snakes on Cocos Island, as well as control snakes in high traffic areas, i.e. vessels that visited Cocos Island daily for business. An awareness campaign targeted staff and visitors to Cocos Island to report sightings and conduct boat inspections. The implementation of personal craft inspections was voluntary with no regulatory enforcement.

In October 2020, a local fisherperson reported killing snakes on Cocos Island during a night-time visit to the atoll. Subsequent search efforts by the U.S. Geological Survey's (USGS) Brown Treesnake Rapid Response Team (RRT; Stanford and Rodda 2007) confirmed a population of brown treesnakes on Cocos Island (Guam Department of Agriculture 2020; Barnhart et al. 2022). The RRT intermittently continued night-time searches through September 2021, with additional training exercises ongoing through September 2023 and had sighted 64 brown treesnakes (58 of which were captured and euthanised; U.S. Geological Survey 2023). As of September 2023, the volunteer group Friends of Islan Dãno had captured and removed 36 brown treesnakes (Martin Kastner, Friends of Islan Dãno, written communication, 2023) and USDA Wildlife Services had removed an additional 23 (Alyssa Taitano, USDA, written communication, 2023). Currently, these search and removal efforts continue intermittently while agencies plan for a more comprehensive response, potentially including an eradication effort (USDA Wildlife Services 2021a, b). Preliminary USGS data reflect an apparently reproductive population, with representatives of all size classes captured and much larger and heavier snakes than found in similar samples from Guam (Barnhart et al. 2022).

Several tools and techniques have been developed and continue to be improved for management of invasive brown treesnakes on Guam (Clark et al. 2018). The common human pharmaceutical acetaminophen (paracetamol) has been identified as an effective oral toxicant for brown treesnakes (Savarie et al. 2000; Siers et al. 2021) with a relatively low environmental risk profile (Johnston et al. 2002). A tablet containing 80 mg of acetaminophen has been registered with the U.S. Environmental Protection Agency as a vertebrate pesticide for brown treesnake control (Reg. No. 56228-34). Coupled with carrion baits (typically 4–6 g dead neonatal mice), acetaminophen baiting has been demonstrated to reduce brown treesnake abundance on a landscape scale (Savarie et al. 2001; Clark and Savarie 2012; Siers et al. 2020a, b), has become a mainstay of interdiction operations (Clark et al. 2012; Clark et al. 2018; Engeman et al. 2018) and is suggested to be capable of eradicating brown treesnakes on Guam within snake barriers, as part of an integrated pest management strategy (Nafus et al. 2022). Baiting can be more cost-effective than traditional trapping methods (Clark et al. 2012) and, as such, was thought to be a desirable eradication tool for managing the established brown treesnake population on Cocos Island.

Since the early 1990s, live trapping with cage traps has been the primary method of brown treesnake removal and continues to be a foundational tool for research and management programmes (Engeman and Linnell 1998; Tyrrell et al. 2009; Clark et al. 2018; Engeman et al. 2018). Current trap designs are modifications of crayfish or minnow traps, composed of a cylindrical wire mesh trap body with a funnel at each end. Stock funnel openings are widened and covered with a wire mesh one-way flap to allow snakes access to the trap body, but blocking escape. A live mouse in a protective chamber is the lure that entices snakes into them. Although these brown treesnake traps are considered the most efficient snake traps in the world (Rodda et al. 1999b), care and provisioning of live mice is costly and infrared photography has indicated that many snakes that encounter traps fail to enter the trap (Yackel Adams et al. 2019).

Moreover, as food resources become more abundant, live mouse traps may have decreasing efficacy, which is of potential importance in rapid response settings (Gragg et al. 2007; Stanford and Rodda 2007). Traps are also expensive and prone to damage by non-target species, particularly coconut crabs (*Birgus latro*) and Mariana monitors (*Varanus tsukamotoi* [nee *indicus*]). Due to these drawbacks, trapping is seen as an effective brown treesnake removal tool, but probably with greatest value when integrated with acetaminophen baiting (Nafus et al. 2022).

It is important to conduct pilot evaluations of the utility of potential control tools to establish their effectiveness prior to substantial investments in planning eradication projects (Genovesi 2001; Clout and Williams 2009). Identifying the limitations of control tools is critical for preliminary feasibility assessments and managing eradication costs. Prior to planning for an eradication attempt on Cocos Island, we sought to evaluate the practicality of acetaminophen baiting and live trapping in the context of the Island's prey-rich environment which is similar to possible scenarios if brown treesnakes were to successfully arrive and establish a population in other areas vulnerable to invasion. Our general objectives for both methods were to evaluate: 1) brown treesnake removal rates with various tool implementation methods; 2) interference by non-target species with brown treesnake removal methods; and 3) potential harm to native species from brown treesnake removal methods.

Methods

Study location

Cocos Island (33.6 ha; Fig. 1) is centred at approximately 13.238°N, 144.653°E and located 2.5 km southwest from the southern coast of Guam, forming part of the Merizo Barrier Reef surrounding Cocos Lagoon. The vegetation is described in detail in Fosberg (1960). The substrate is deep, well-drained loamy sand and the flora is primarily *Casuarina equisetifolia* forest in the northeast, while the south-western portion of the island is primarily mixed strand forest comprising *Cocos nucifera*, *Hernandia sonora*, *Guettarda speciosa*, *Merrilliodendron megacarpum*, *Morinda citrifolia*, *Intsia bijuga*, *Casuarina equisetifolia*, *Terminalia catappa*, *Tournefortia argentea*, *Carica papaya*, *Barringtonia asiatica*, *Hibiscus tiliaceus*, *Leucaena leucocephala* and *Thespesia populnea*. Vegetation along the south-eastern shore is dominated by *Pemphis acidula* and *Scaevola sericea*, while the north-western coastline is mostly open sand.

The north-eastern 80% of the Island is under private ownership and the south-western 20% is owned by the Government of Guam and managed by the Guam Department of Parks and Recreation. The Island is uninhabited, but Cocos Island Resort operates as a day resort offering water-sports, trail walks and food and beverages. The resort closed when the Governor of Guam declared an island-wide public emergency shutdown in response to the COVID-19 pandemic on 13 March 2020. The resort has not yet reopened since then.



Figure 1. Map of Cocos Island 2.5 km from the southern tip of Guam, USA. Orange lines indicate locations of trails used for bait applications and trapping. The top of the image is orientated to the north. Image: Maxar Intelligence 2021.

To minimise disturbance of threatened and endangered species present on the Island, our activities were limited to the edges of existing cart paths and footpaths as per the conditions of our U.S. Fish and Wildlife Service Endangered Species Act consultation (Fig. 1). Cart paths and trails on Cocos Island receive very little maintenance or traffic and are primarily under continuous canopy and do not substantially alter the surrounding forest structure. The majority of mainland Guam brown treesnake research is conducted along road edges and maintained transects (e.g. Christy et al. (2010); Siers et al. (2017b)) so we believe it unlikely that limiting activities to these areas would bias results.

Acetaminophen baiting

We sought to evaluate the relative merits of a variety of potential baits and bait presentation methods on Cocos Island. Preliminary evidence from mainland Guam indicates that brown treesnakes with recent experience feeding on birds may be preferentially attracted to dead bird baits over dead rodents (Nafus et al. 2021). To evaluate bait

preferences, we offered three sizes of dead mice and two sizes of dead bird chicks as baits, which were suitable to the presentation method: 4–6-gram dead neonatal mice (DNM); 10–17-g small mice (SM); 18–35-g large mice (LM); 10–14-g small bird baits (hatchling quail, SB); and 25–35-g large bird baits (hatchling chickens, LB) (Fig. 2). All baits were monitored with commercial infrared game cameras (H68, Ape-man, Shenzhen, China) set to time-lapse with one image recorded every 30 seconds for 24 hours per day.



Figure 2. Dead animal baits used in this study. Left to right: 4–6-g dead neonatal mouse (DNM); 10–17-g small mouse (SM); 18–35-g large mouse (LM); 10–14-g small bird (quail chick, SB); and 25–35-g large bird (chicken chick, LB).

Canopy presentation: USDA Wildlife Services has engineered an Aerial Delivery System (ADS) for the automated assembly and aerial distribution of bait cartridges containing a DNM treated with a tablet containing 80 mg of acetaminophen (Siers et al. 2019a, 2020b, 2021; Goetz et al. 2020, 2021). These cartridges open upon ejection from the aircraft, exposing a ribbon to cause entanglement in the forest canopy where arboreal treesnakes forage, preventing baits from falling to the forest floor where they can be taken by terrestrial non-target species, such as crabs. We emulated aerial bait applications by positioning opened ADS cartridges on a simulated branch (45-cm wooden dowel) with the DNM hanging in the field of view (FOV) of a camera mounted atop a painter's pole, extended into the forest canopy and temporarily lashed to natural vegetation with bungee cords (Fig. 3A).

Ground presentation: As some ADS baits fail to tangle in the canopy or DNM may become unstuck from the cartridge and fall through to the forest floor, we sought to evaluate the fate of DNM on the ground. Additionally, recent evidence



Figure 3. Camera orientations for canopy and ground bait monitoring **A** infrared camera set-up mimicking aerial application of dead neonatal mouse (DNM) baits via the USDA Wildlife Services Aerial Delivery System (ADS) for landscape-scale brown treesnake control; the camera and bait are elevated into the forest canopy atop a telescoping painter's pole **B** ground bait monitoring set-up, with an infrared camera mounted directly over a large mouse (LM) bait on a tripod constructed from extruded metal tubing (conduit); the bait and a lightweight PVC background with circular size standards were lashed to a 0.9-kg lead diving weight to prevent small crabs from dragging the bait out of the camera's field of view.

indicates that ADS treatments might not adequately expose large brown treesnakes to baits; larger brown treesnakes on prey-depleted Guam are more prone to foraging on the ground, might be preferentially attracted to larger baits and might require greater doses of acetaminophen for effective removal (Rodda and Reed 2007; Nafus et al. 2020; Goetz et al. 2021; Siers et al. 2021). Moreover, the estimated take rates for large ground baits in a treated population on Guam is greater than for standard ADS baits (Nafus et al. 2022). For these reasons, USDA Wildlife Services has considered an alternative aerially delivered bait system comprising a slightly larger mouse (SM) placed in the same bait cartridge tube, but without the ribbon assembly, which would let the bait fall to the forest floor where larger snakes may be more effectively targeted (Siers et al. 2021). We also considered that even larger baits placed on the forest floor, potentially containing larger doses of acetaminophen, could more effectively target larger ground-foraging snakes, so we incorporated LM and LB into ground presentations. Ground baits were placed on small, thin PVC plastic platforms printed with 20-cm size standards and lashed to a 0.9-kg. lead diving weight to prevent small crabs from dragging the bait out of the field of view of the cameras. Cameras were mounted directly overhead on tripods fashioned from inexpensive extruded metal tubing (conduit; Fig. 3B). Brown treesnake head measurements taken from overhead images containing a size standard can be used to estimate snake size (Siers 2021). As both canopy and ground baits are not protected from being taken by non-target species, we did not treat these baits with acetaminophen tablets for this pilot evaluation.

Bait station presentations: Polyvinyl chloride (PVC) tube bait stations ('bait tubes') are intended to exclude non-target species that might interfere with baits, making them unavailable to brown treesnakes and to protect native species from unintentional exposure to acetaminophen intoxication. Standard operational baiting methods include placing a DNM treated with a tablet containing 80 mg of acetaminophen into a 5-cm diameter, 30-cm long PVC bait tube, with 6.35-mm bolts crossing the openings at the ends to further prevent ingress by non-targets. Bait tubes are usually suspended horizontally by two lengths of paracord from existing vegetation or structures, such as fence lines (Savarie et al. 2001; Clark et al. 2012; Lardner et al. 2013; Clark et al. 2018). Based on the average size of brown treesnakes recovered from Cocos Island prior to the testing of these tools (Barnhart et al. 2022), we elected to deploy larger baits (SM, LM, SB and LB) within our bait stations on Cocos Island. We evaluated standard 5 × 30-cm horizontal bait tubes (Fig. 4A), as well as alternative designs intended to more reliably exclude non-target species, such as crabs and Mariana monitors (e.g. Mathies et al. (2011)): longer 5 × 45-cm horizontal bait tubes (Fig. 4B); vertical 5-cm diameter × 30-cm long bait tubes capped at the top end (Fig. 4C); and capped vertical 10-cm diameter × 30-cm long bait tubes (Fig. 4D). Both horizontal bait tube designs included bolts across the openings, whereas vertical tubes did not to prevent use of the bolts by non-targets to assist climbing into the tubes.

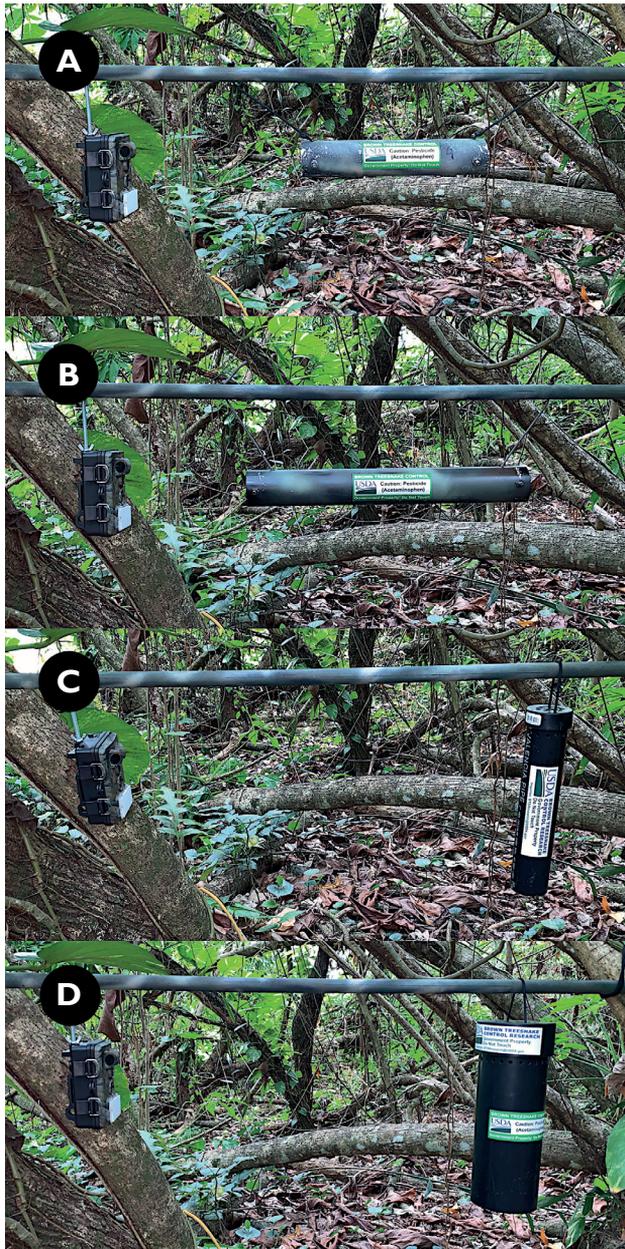


Figure 4. Camera orientations and bait station configurations for bait monitoring **A** standard 5-cm diameter \times 30-cm long polyvinyl chloride (PVC) horizontal bait station suspended by nylon paracord from a wooden dowel armature with infrared camera positioned with the bait in the field of view; the two ends of the armature are temporarily lashed to natural vegetation with elastic cords **B** extended 5 \times 45-cm horizontal bait tube **C** capped 5 \times 30-cm vertical bait tube **D** capped 10 \times 30-cm vertical bait tube. Baits in horizontal bait tubes were held in place by gravity, while baits were held in the caps of vertical bait tubes by spring clamps on one foot of the bait. As baits in vertical bait tubes were not visible to the camera, a length of biodegradable flagging was tied to one leg of the bait and pulled through a small hole in the side of the tube for ease of identifying when the bait was taken during camera image review.

We suspended all bait station types by paracord from a wooden dowel armature to which the trail camera was affixed, locking the bait in the FOV of the camera. We temporarily attached these armatures to existing vegetation by elastic cords, with minor vegetation pruning to ensure that leaves or branches did not obstruct the view of the bait. We placed baits in the centre of horizontal tubes where they were held in place by gravity and friction alone. Within vertical bait tubes, we attached baits within the caps clipping one foot with a small metal spring clamp, holding baits in place, but making them easily removed with a slight tug from snakes or non-targets. As cameras could not directly view baits in the vertical tubes, we tied a length of white biodegradable flagging tape to one leg of each bait and pulled the tape through a small hole in the side of the tube; when the bait was removed, the flag disappeared and the animal within the FOV of the camera at that time was attributed with the bait removal.

As bait stations offered some degree of protection from non-target interference, we treated baits in bait stations with tablets containing 80 mg of acetaminophen inserted into the body of the bait via the oral cavity, to remove any brown treesnakes that took baits.

We spaced bait placements at approximately 20-m intervals along existing paths and trails on Cocos Island (Fig. 1); entry into the forest was not authorised prior to a formal endangered species consultation process with the U.S. Fish and Wildlife Service. Paths were subdivided into nine, segments (transects). Each week we placed 36 bait monitoring stations along one transect, alternating each of three presentation types (12 stations each). We monitored each transect of 36 stations for one week, with the bait checked and replaced once mid-week with a fresh bait, for baiting intervals of 3 to 4 days. Beyond 3 to 4 days, baits are degraded through putrefaction and consumption by ants and fly larvae and are no longer considered viable for take by brown treesnakes. During Weeks 1–9 (12 December 2020–16 February 2021), we placed canopy, ground and standard 5 × 30-cm horizontal tubes (Figs 3A, B, 4A) in alternating positions along each transect, with bait types alternated as appropriate to the objectives of the presentation type. During Weeks 10–18 (18 February 2021–22 April 2021), we alternated 5 × 45-cm horizontal tubes and the two vertically orientated tube types (5 × 30 and 10 × 30 cm; Fig. 4B–D), along with alternating bait types. At bait checks, if a bait appeared to have been taken, we reviewed camera images to identify the time of bait removal and the species taking the bait.

This portion of the study was performed during Guam's cooler, drier months. Average daily temperatures for Guam ranged from highs of 30.4 °C (standard deviation = 0.903 °C) to lows of 24.7 °C (SD = 1.03 °C) and rainfall averaged 2.16 mm/day (SD = 5.3 mm/day, max = 48 mm/day), based on National Oceanic and Atmospheric Administration data (www.weather.gov).

Live trapping

After years of experimentation with multiple live trap designs, a modified crayfish or minnow trap was adopted as the standard brown treesnake live trap used on Guam. The original trap is a two-piece dual-funnel design of galvanised wire mesh with the entrances modified with a PVC ring holding a one-way wire mesh flap that allows ac-

cess to the trap body, but blocks escape by snakes (Fig. 5, left). Rodda et al. (1999b) showed this trap to be the most effective trap known for any snake at the time. Snakes are lured into the traps by a live mouse in a protected wire mesh chamber held within the trap body. This two-piece design was later adapted for operational purposes by creating a single-piece body of more durable stainless steel, accessed by removing one of the funnel ends and incorporating the mouse chamber into the trap body so that the mouse can be serviced without opening the trap; this version of the trap is referred to as the USDA Wildlife Services “WS Standard” (Fig. 5, centre; Vice et al. (2005)). In both trap types, mice are provisioned with a custom-made block of commercial seed and pellet mix embedded in a paraffin wax matrix which prevents exposure to the elements until the mouse chews through the wax (Fig. 5, right). A piece of fresh potato provides the necessary water.

The use of live mouse lures is less than desirable due to maintenance expense and perceptions regarding animal welfare; however, despite extensive efforts, no trap lure has been found to be nearly as effective and practical as a live mouse (Chiszar 1990; Shivik and Clark 1997; Shivik 1998, 1999; Lindberg et al. 2000). Prior to verification of brown treesnake presence on the Island, Guam’s Division of Aquatic and Wildlife Resources (GDAWR) performed surveillance trapping using dead mouse and rat lures due to concerns about escaped live mice establishing a population on the Island (D. Vice, GDAWR, written communication, 2023) but no snakes were ever captured in these traps. Prior to this study, there were no reported head-to-head tests of live versus dead mouse lures in brown treesnake traps.

To evaluate differences in efficacy and durability between trap types and capture success between live and dead mouse lures, we alternated 99 one-piece WS Standard and 99 original two-piece traps approximately every 20 m along the same existing trails as the previous baiting trials (Fig. 1). We alternated live and dead mouse lures between



Figure 5. Two types of traps used. Left: Galvanised wire mesh two-piece trap with separate live mouse lure chamber within the trap body. Centre: Stainless steel one-piece Wildlife Services Standard trap with integrated live mouse lure chamber. Right: Integrated lure chamber in one-piece trap showing live mouse, feed block of pellets and seeds immersed in paraffin wax and piece of fresh potato to provide moisture; mice in both trap types are provisioned in this manner.

every pair of two trap types (repeating the order of one-piece/live, two-piece/live, one-piece/dead, two-piece/dead etc.) for a total of 100 traps with live mouse lures and 98 with dead mice. Following the typical use patterns of these two trap types, we hung one-piece traps on nylon paracord and two-piece traps on metal tie wire, at about waist to chest height on existing vegetation. We checked traps twice weekly (every 3 or 4 days) for 55 nights, provisioning live mouse lures and replacing dead mouse lures with fresh dead mice. We recorded brown treesnake captures, non-target captures and trap damage caused by non-target species. We also recorded traps as non-functional when missing lures, with funnel flaps stuck open or closed, with large holes due to crab damage or with other defects making them unlikely to capture or prevent escape by snakes. As traps were confirmed to be functional at the beginning of each trap-checking interval, we assumed traps became non-functional approximately mid-interval, on average, so reduced our tally of effective trap nights by one-half of the checking interval per non-functioning trap, similar to methods of Nelson and Clark (1973). Trapping results are reported as captures per unit effort (CPUE) or snake captures per night per trap.

We monitored a subset of 20 traps via infrared game cameras (Hyperfire 2, Reconyx, Holmen, Wisconsin). We distributed 10 cameras evenly along a rock retaining wall and another 10 along a transect through a bird roosting area. We positioned half of these cameras on traps with live mouse lures and the other half on traps with dead mice. We recorded time-lapse images (one photo every 60 seconds) between 1800 and 0600 h to observe for brown treesnakes investigating traps, but failing to enter, as has been documented on mainland Guam (Yackel Adams et al. 2019; Amburgey et al. 2021).

We performed trapping from 17 June to 12 August, 2021, earlier months of Guam's warmer, rainier season. Guam daytime highs averaged 31.4 °C (SD = 1.04) with night-time lows of 25.6 °C (SD = 0.969) and rainfall of 9.22 mm/day (SD = 13.5mm, max = 71.9mm) (www.weather.gov). We measured snout-vent length (SVL, mm) of trapped snakes by gently stretching them along a flexible tape and measured weight using handheld spring scales with maximum ranges from 10 g (0.1 g precision) to 1000 g (10 g precision) (Pesola, Schindellegi, Switzerland). We determined sex by probing for inverted hemipenes with steel sexing probes (Reed and Tucker 2012).

Visual detection during rapid response

Throughout the evaluation of control tools, USGS conducted nocturnal visual searches and hand-removal of brown treesnakes (December 2020 through July 2021). Methods followed those applied on Guam in which individuals surveyed transects after dusk using powerful headlamps (Wilma, Lupine Lighting System, Lebanon, PA, USA) walking a slow searcher pace, such that each transect (~ 400 m) lasted approximately 1 hour. During snake searches, observations of potential prey items (lizards, birds and bats) were recorded. Visual survey data are available for download (U.S. Geological Survey 2023). These searches were also limited to the cart paths and trails depicted in Fig. 1. We recorded SVL, weight and sex for captured snakes as above.

Statistical methods

All summary statistics, statistical tests and graphing were performed in the R environment for statistical computing, Version 4.2.2 (R Core Team 2021). We evaluated the likelihood that an unobserved bait take due to camera malfunction could have been taken by a snake by describing the 95% confidence interval of brown treesnake bait takes given the successfully observed baits (`binom.confint`, `method = "exact"`). We evaluated differences in duration of bait availability amongst presentation types with Cox proportional hazard survival models function (`coxph`), with trials ending when the bait was taken by a non-target or when the three to four days monitoring period was over. Differences in trap capture rates amongst trap types and trap lure types were calculated using Fisher's exact tests with 95% confidence intervals (`fisher.test`).

Ethics statement

This study was carried out in compliance with relevant laws and guidelines. All animal use was approved by the USDA National Wildlife Research Center Institutional Animal Care and Use Committee under protocols QA-3106 and QA-3340 and USGS Institutional Animal Care and Use Committee protocol 2021-02. Compliance with the Endangered Species Act was ensured through informal consultation with the U. S. Fish and Wildlife Service (01EPIF00-2021-I-0087 and 01EPIF00-2021-I-0087-R001).

Results

Acetaminophen baiting

After eliminating incomplete trials (camera failure etc.), we successfully monitored a total of 1,250 baits between December 2020 and April 2021. During these trials, we observed no baits being investigated or taken by brown treesnakes. As there were no bait takes by brown treesnakes, we could not make comparisons of bait take rates amongst bait types or presentation types. Of the 701 baits that were taken, we could not identify the species in 30 (4.3%) of the cases. The 95% binomial confidence interval for brown treesnake takes for the 671 baits for which a species ID was confirmed is 0–0.548%; if this rate were applied to the 30 unknown takes, the upper confidence limit for brown treesnake bait takes would be 0.164 baits; thus, we consider it highly unlikely that any of the unknown animals taking these 30 baits was a brown treesnake.

A high overall proportion of the baits (56.1%) were taken by non-target species (Fig. 6), primarily by coconut crabs and Mariana monitors. Canopy baits were removed by coconut crabs (11.5%), Mariana monitors (12%) and insects (11.5%, mostly ants and fly larvae), while 20.7% fell from the simulated bait cartridge due to putrefaction or consumption by insects. One bait was removed from the bait cartridge by a large gecko (*Gehyra oceanica*). Of the DNM baits in the canopy, 43.8% remained available (i.e. were not taken by non-targets), but were not observed being investigated or taken by brown

treesnakes. All but three baits placed directly on the ground were taken by non-targets, mostly coconut crabs and Mariana monitors, with some take by hermit crabs (*Coenobita* spp.) and land crabs (likely *Cardisoma carnifex* or *Discoplax* spp.). One DNM ground bait was taken by a Pacific reef heron (*Egretta sacra*) and another by a Guam rail. Of baits offered in the standard 5 × 30-cm horizontal tubes, 66.3% were taken by Mariana monitors and coconut crabs. The other bait station designs successfully repelled almost all coconut crabs; Mariana monitors continued to be the primary challenge for all bait station types, but the 5 × 45-cm horizontal tube reduced monitor takes to only 15.8% (Fig. 6).

Coconut crabs and Mariana monitors were by far the most common consumers of baits. Plotting the recorded time of bait takes by coconut crabs and Mariana monitors (Fig. 7) reflected clear patterns of nocturnal activity for coconut crabs and diurnal activity for Mariana monitors, with bimodal peaks of monitor activity in early morning and late afternoon.

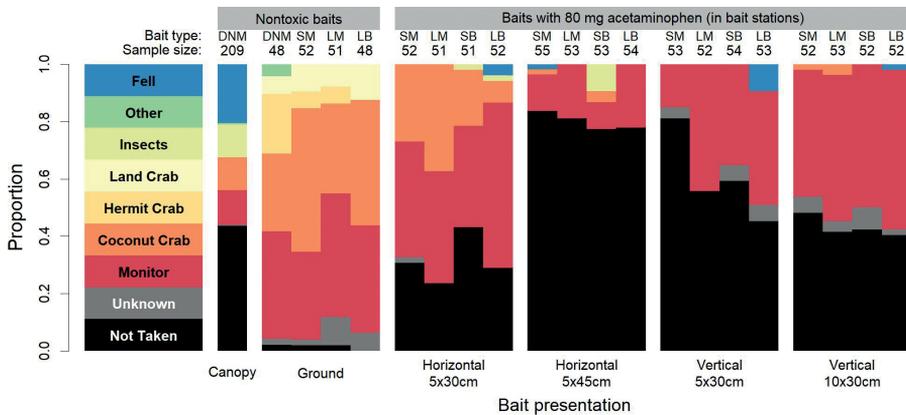


Figure 6. Fates of carrion baits applied on Cocos Island. No brown treesnakes were observed taking any baits. DNM = dead neonatal mouse (4–6 g); SM = small mouse (10–14 g); LM = large mouse (25–35 g); SB = small birds (10–14 g quail chick); LB = large bird (25–35 g chicken chick). Sample size is the number of baits successfully monitored after eliminating incomplete trials.

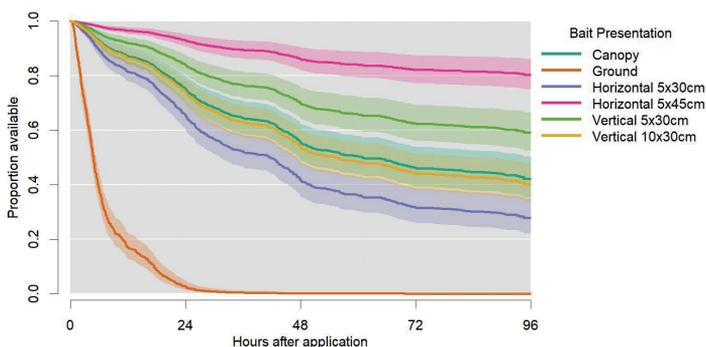


Figure 7. Times of bait removal by the primary non-target consumers on Cocos Island. Most baits were removed by coconut crabs (*Birgus latro*) and Mariana monitors (*Varanus tsukamotoi*). Light grey strips indicate changes in sunrise and sunset over the study period.

We recorded the duration of bait availability before being taken by non-targets or removed at the end of the trial and subjected these data to survival analysis (Fig. 8). Non-targets removed almost all ground baits within 24 hours. The horizontal 5 × 45-cm bait tube clearly outperformed all other presentation types, with 80% of the baits remaining at the end of the observation period.

Summing all the time that baits were available to brown treesnakes before being taken by non-targets, we recorded a total of 2,427 “bait days” with no takes by brown treesnakes. This amounts to an overall daily estimated brown treesnake bait take rate of 0.000 per 100 bait days with an upper 95% binomial confidence interval of 0.151 baits per 100 bait days.

Trapping

We operated 198 traps (99 one-piece, 99 two-piece) with mouse lures (100 live, 98 dead) for 55 nights, for a total of 10,890 trap nights (Table 1) between and June and

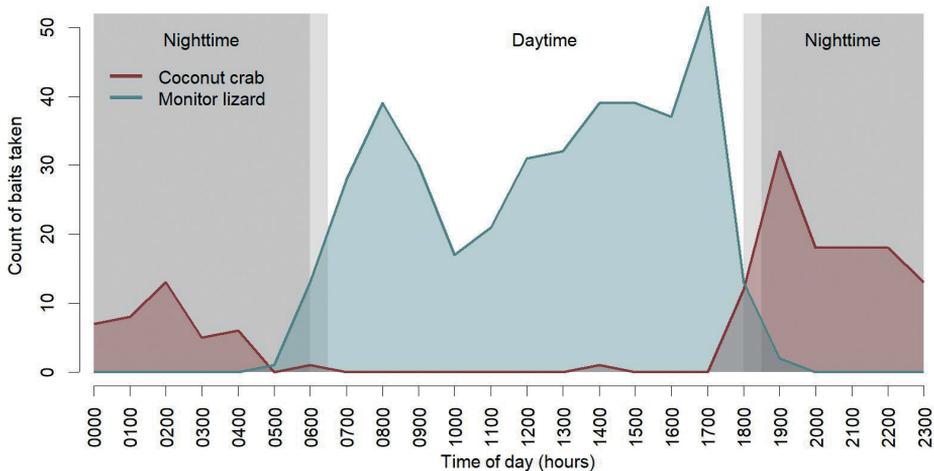


Figure 8. Persistence of all baits offered in various presentation types. Curves depict reduction in proportion of baits available over time as non-targets remove them. Baits typically decline in viability through putrefaction and consumption by insects after 48 to 96 hours. Shaded areas represent 95% confidence intervals.

Table 1. Trapping effort (trap nights) by trap type and lure type. Values reflect overall effort and effort adjusted for non-functioning traps (e.g. lure missing, entrance flaps stuck open or closed, holes due to crab damage). Traps were functional at the previous check, so non-functional traps were presumed to be functional for one-half of the check interval, on average.

Trap type	Overall effort (trap nights)			Adjusted for non-functioning traps		
	Live mouse	Dead mouse	Total	Live mouse	Dead mouse	Total
One-piece	2,750	2,695	5,445	2,682.5	2,620.0	5,302.5
Two-piece	2,750	2,695	5,445	2,663.0	2,588.0	5,251.0
TOTAL	5,500	5,390	10,890	5,345.5	5,208.0	10,553.5

August of 2021. After adjusting for non-functioning traps, we achieved an effort of 10,553.5 effective trap nights.

During this effort, we captured only one brown treesnake: a 1,249-mm SVL male weighing 360 g (Fig. 9). The snake was captured in a two-piece galvanised trap with a live mouse lure. One capture in 10,553 trap nights yields a combined CPUE of 9.47×10^{-5} or 0.00947 captures per 100 trap nights. Considering CPUE only for traps with live mouse lures increased CPUE to 0.0187 captures per 100 trap nights. The 95% binomial confidence interval for trap success (probability of each trap capturing at least one snake on a given night) with a live mouse lure is 0.000473 to 0.0528 trap successes per 100 trap nights.

Non-target lizards and crabs were commonly found in brown treesnake traps (Table 2). As with acetaminophen baiting, coconut crabs and Mariana monitors were the most significant non-targets, being large and powerful and capable of damaging traps. Smaller lizards were more common in traps with dead mouse lures, likely drawn by flies and other insects feeding on the carrion lure. Crabs, including coconut crabs, were more often found in the one-piece WS standard traps, possibly because one-piece traps were hung with nylon paracord, per WS standard operations, while two-piece traps were hung by metal tie-wire; it is conceivable that metal wire provides less purchase for crabs attempting to access traps. Crabs were more prevalent in traps with live mouse lures, probably attracted by feed waste and faeces generated by the mouse.



Figure 9. The only brown treesnake captured in over 10,500 trap nights on Cocos Island. This was a 1,249 mm snout-vent length, 360 g, male. This trap contained a live mouse in a protected chamber as the lure.

Table 2. Non-target captures in brown treesnake traps. Results are tabulated by trap type (one-piece stainless steel versus two-piece galvanised) and lure type (live mouse or dead mouse). Counts are per trap observation; multiple individuals in the trap at the same time are counted as only one observation.

Common name	Latin name	One-piece Live	Two-piece Live	One-piece Dead	Two-piece Dead	Total
Lizards						
Green anole	<i>Anolis carolinensis</i>	1	0	2	3	6
Blue-tailed skink	<i>Emoia caeruleocauda</i>	0	0	1	0	1
Oceanic gecko	<i>Gehyra oceanica</i>	0	0	2	0	2
Other geckos	Gekkonidae	1	1	1	1	4
Mariana monitor	<i>Varanus tsukamotoi</i>	8	11	14	5	38
Crabs						
Hermit crabs	<i>Coenobita</i> spp.	183	24	14	1	222
Coconut crab	<i>Birgus latro</i>	57	9	22	17	105
Totals		250	45	56	27	378

Stainless steel one-piece traps were non-functional for 5.2% of trap nights, while galvanised two-piece traps were non-functional for 7.1% of nights ($P < 0.001$). Live mouse lure traps were non-functional 5.6% of trap nights and dead mouse lure traps were non-functional 6.8% of nights ($P = 0.015$). Stainless steel one-piece traps required 21 field repairs for a total of 2.83 hours of labour, while galvanised two-piece traps required 82 repairs totalling 12.4 hours of field labour. Only one trap, a two-piece galvanised trap, was removed for workshop repairs. At the end of the trapping effort, repair and cleaning requirements were recorded by trap type (Table 3). The one-piece stainless steel traps were more durable to crab damage and required less labour to fix, primarily straightening deformations of unbroken wire mesh, while all damaged two-piece galvanised wire traps required more serious repairs, such as hole patches. No traps of either type were damaged beyond repair. Although galvanised wire traps are likely more prone to corrosion than stainless steel over prolonged use in the near-marine environment, there was little noticeable corrosion over the course of this study. The one-piece traps required more time to clean due to the lure chambers being integrated into the trap body.

Table 3. Repair and cleaning requirements by trap type. Minor repairs included straightening wire deformations from coconut crab damage, while major repairs required patching of holes in the mesh from crab damage.

Trap type	No repair (n)	Minor repair (n)	Major repair (n)	Repair labour (hr)	Cleaning labour (hr)
One-piece stainless steel	28	47	24	13	20
Two-piece galvanised wire	31.5*	0	67.5	27	7
Totals	59.5*	47	31.5	40	27

* The two-piece traps were recorded by interchangeable trap halves (two halves = one trap), hence the 0.5-trap increments.

During 1,100 trap nights monitored by infrared cameras (20 cameras, 647,733 total photos), we recorded only two trap encounters by brown treesnakes, of 2 and 8 minutes in duration (Fig. 10). In neither of these cases did the snake successfully enter the trap. Both recorded encounters were of a similarly-sized snake on the same trap with a live mouse lure 10 nights apart; we believe it reasonable to consider these to be two observations of the same snake.

Visual detection during rapid response

Throughout the time period of our baiting and trapping efforts (December 2020 to July 2021), USGS personnel performed 163.3 km of linear search effort over 376 hours and sighted 31 snakes, three of which escaped capture, for a visual detection rate of 0.188 snakes per km and 0.083 per hour of search. Prey sighting rates were high, with an average lizard sighting rate of 37.8/km (including 10 species) and 32.8 birds/km (8 species). Specifically for notable species, sighting rates were 15.5 green anoles, 11.3 oceanic geckos, 22.3 black noddies and 8.1 white terns per km of searching (U.S. Geological Survey 2023). The mean SVL of snakes captured and removed was 1073 ± 295 (range: 650, 1622) and mean weight was 256 ± 195 g. The demography of the population encountered was biased towards snakes typically attracted to endothermic prey (60% of 28 captures > 900 mm SVL).



Figure 10. A large brown treesnake at a two-piece trap with a live mouse lure recorded via time-lapse infrared photograph. The snake probed the body of the trap for 8 minutes, then left without returning that night. A snake of a similar size (likely the same snake) attempted to enter the same trap for 2 minutes 10 days later.

Discussion

The results of this study are clear: two of the primary tools for brown treesnake removal on Guam will not be effective for brown treesnake eradication on Cocos Island. Brown treesnake detection rates, based on visual CPUE, are apparently lower on Cocos Island (0.188 snakes/km) than most other detection efforts on Guam. Within a long-term 5-ha geographically enclosed population representative of disturbed limestone forest and secondary forest on Guam, Nafus et al. (2023) recorded 0.758 brown treesnakes per km of transect searched. In a 55-ha snake enclosure surrounding degraded limestone forest in northern Guam, Boback et al. (2020) documented a CPUE of 0.906 brown treesnakes per searcher hour, compared to 0.083 on Cocos Island. Following aerial baiting at this same site (Dorr et al. 2016; Siers et al. 2018), the CPUE dropped to 0.049 per hour, lower than the Cocos Island CPUE. With all of the caveats that come with using CPUE as an index of relative abundance (e.g. detectability differences amongst different habitat types), these data demonstrate that visual detection rates on Cocos Island are lower than unmanipulated habitat on Guam (24% compared to Nafus et al. (2023) per km and 9.2% compared to Boback et al. (2020) per hour before aerial suppression), but nearly twice as high as within an aeriually-suppressed study plot (Boback et al. 2020). Nonetheless, despite 40% lower visual contact rates than on Cocos Island, the suppressed population on Guam continued to take non-toxic dead mouse baits at rates averaging approximately 20% (Siers et al. 2018b), while the Cocos Island bait take rate was 0%.

Live trapping with mouse lures prior to snake suppression on Guam yielded a capture rate of 0.3 snakes per 100 trap days (Nafus et al. 2018). These differences in snake detection rates by location indicate that greater bait or trap captures on Cocos Island would be expected given the level of effort we applied to each tool. Carrion bait take rates on Guam in areas without active brown treesnake control tend to range from approximately 30% to nearly 100% (Savarie et al. 2001; Clark and Savarie 2012; Siers et al. 2018b, 2019b, 2020a), while we failed to observe a single bait take within 2,427 bait monitoring days (upper 95% confidence interval of 0.151%). Trapping captures per 100 trap nights on Guam are commonly higher in areas where they are not being operationally suppressed, (4–9; Nafus et al. (2018)) and ranged as high as 25 to 60 in the 1990s (Rodda et al. 1999b). In areas on Guam in which brown treesnakes have been suppressed to 0.16 snake/ha (as estimated from forest interior visual survey CPUE), trapping CPUE was maintained at the rate of 0.3 captures per 100 trap nights (Nafus et al. 2018; Boback et al. 2020). When using live mouse lures on Cocos Island, we achieved only 0.0187 captures per 100 trap nights which is substantially lower than the anticipated levels based on Guam efforts (Guam CPUE 213 to 481 times higher than Cocos Island when compared to Nafus et al. (2018) results).

Our failure to attract brown treesnakes to baits and traps is most likely due to the extraordinary abundance of preferred live prey on Cocos Island, particularly large geckos, birds and their eggs, compared to the relatively prey-depauperate nature of Guam's forests resulting from prolonged brown treesnake predation (Fritts and Rodda

1998; Wiles et al. 2003; Siers 2015). Cocos Island is populated by abundant lizards, many of which have been extirpated from parts or all of Guam by brown treesnake predation, such as federally and locally endangered Mariana skinks, locally endangered littoral skinks (*Emoia atrocostata*), fragile Micronesian geckos (*Perochirus ateles*) and Pacific snake-eyed skinks (*Cryptoblepharus poecilopleurus*), as well as mutilating geckos (*Gehyra mutilata*), oceanic geckos (*G. oceanica*) and green anoles (*Anolis carolinensis*). Small brown treesnakes, in particular, appear to be specialists on small lizards, which are an almost exclusive prey item in stomach contents (Savidge 1988; Siers 2015). They also exhibit strong preference during laboratory feeding trials (Lardner et al. 2009) and present a venom composition that is more effective for ectotherms as juveniles (Mackessy et al. 2006). Our green anole sighting rates were relatively high (15.5/km), but close to estimates from the snake-free island of Saipan (14.9/km; Lardner et al. (2019a)), while observations of this species in brown treesnake stomach contents from mainland Guam are extremely low in forest habitats, from where they have been essentially extirpated by brown treesnake predation (Rodda and Fritts 1992; Siers 2015). Relatively large oceanic geckos, which are roughly equivalent in mass to a small mouse, may additionally offer a rewarding prey item that reduces the efficacy of bait-and lure-based control tools on Cocos Island. We commonly observed oceanic geckos on Cocos Island (11.3/km), while they are no longer documented in brown treesnake stomach contents on mainland Guam (Siers 2015), having been essentially extirpated by brown treesnake predation (Rodda and Fritts 1992). Nafus et al. (2023) recorded no observations of green anoles or oceanic geckos and their total lizard sighting rates were 6.0/km (4 species) compared to 37.8/km (10 species) on Cocos Island, indicating substantially greater lizard prey availability. Unchecked brown treesnake predation and population growth could put all small lizard species at risk of almost certain local extinction on Cocos Island (Rodda and Fritts 1992; Rodda et al. 1997; Fritts and Rodda 1998; USFWS 2019).

Of all the ecological damage that have occurred since their introduction to Guam, the pervasive impacts of brown treesnake predation on birds have been the most profound (Savidge 1987; Wiles et al. 2003; Pollock et al. 2019; Klug et al. 2021b). Our Cocos Island bird sighting rate of 32.8/km and 11.3/hour is high, given the well-documented collapse and functional extinction of the forest bird avifauna on mainland Guam (Savidge 1987; Wiles et al. 2003). Any experienced brown treesnake searcher can attest that bird sightings in forest habitats on mainland Guam are quite rare, earning Guam its reputation for “silent forests” and the cascading ecological effects of bird loss (Savidge 1987; Rodda et al. 1997; Rogers 2011, 2020). Nafus et al. (2023) documented only four bird sightings of indeterminate species in 816.2 km of transect searching in the northern Guam 5-ha enclosure (0.005 birds/km). It is unclear how severely brown treesnake predation on Cocos Island has affected the abundance of bird and lizard prey species, but prey resources clearly remain much more abundant on Cocos Island.

Brown treesnakes on Guam that forage in areas of increased prey availability, including birds, such as urban areas and swiftlet caves, tend to be in better body con-

dition (Siers 2015; Siers et al. 2017b; Yackel Adams et al. 2019; Klug et al. 2021b), a characteristic that is evident in the extremely heavy snakes that have been found during visual surveys on Cocos Island. The mean weight of snakes reported in other contemporary studies on Guam (Siers et al. 2017b) suggests that the average weight of snakes removed from Cocos Island during the period of this study is much greater. There is also emerging evidence that brown treesnakes conditioned to feeding on live birds exhibit less attraction to rodent-based baits and lures (Nafus et al. 2021). During brown treesnake removal from Cocos Island, encounters with seabirds, Guam rails and Micronesian starlings (*Aplonis opaca*) were not uncommon (U.S. Geological Survey 2023), supporting the impression that avian prey currently remain abundant on Cocos Island relative to Guam. For these reasons, it is apparent that, although brown treesnakes on Guam will readily consume carrion, live lures are far more effective than any dead animal baits or other inanimate lures (Shivik et al. 2000; Savarie and Clark 2006; Kimball et al. 2016) and that the availability of abundant preferred prey (birds) on Cocos Island diminishes the attraction to carrion baits.

These findings demonstrate that higher prey availability negatively affects brown treesnake detection and capture rates. On Guam, temporary experimental suppression of rodent prey abundance was demonstrated to increase trap capture rates (Gragg et al. 2007) and increasing movement distances of brown treesnakes (Christy et al. 2017). Free-ranging brown treesnakes that have recently taken large meals have been experimentally demonstrated to significantly reduce movement for 5 to 7 days, with an associated reduction in the ability to visually detect or trap snakes during this time (Siers et al. 2018a). Foraging for carrion may also be a futile strategy for brown treesnakes on Cocos Island where coconut crabs and Mariana monitors rapidly remove all carrion from the ground (Figs 7–9). Moreover, prior studies have indicated that brown treesnake attraction to carrion and especially mouse carrion, may decrease as snakes increase in size (Shivik and Clark 1999; Nafus et al. 2021), while large snakes may be the most important demographic to remove from the perspective of both avian conservation and eradication potential (Savidge 1988; Yackel Adams et al. 2019; Nafus et al. 2022).

Both baiting and trapping appear to be relatively safe for Cocos Island wildlife. In only two instances did a native bird (a Pacific reef heron and a Guam rail) take a bait (both DNM) from the ground. Although acetaminophen may also be toxic to birds, they are not known to have the same genetic basis for sensitivity to acetaminophen toxicosis as snakes (van den Hurk and Kerckamp 2019) and crows in cage trials picked around acetaminophen tablets rather than ingesting them, with no signs of toxicosis (Avery et al. 2004). Clearly, crabs and Mariana monitors quickly cleanse the forest floor of any carrion baits containing acetaminophen. Crabs tend to eat around acetaminophen tablets when consuming carrion baits and show no signs of toxicosis (Johnston et al. 2002). On the other hand, Mariana monitors were the most problematic scavengers of baits on the ground and in bait stations and other monitor species are susceptible to acetaminophen toxicosis (Mauldin and Savarie 2010). Although recent evidence indicates that Mariana monitors are native to the Mariana Islands (Weijola et al. 2020), Mariana monitors on Cocos Island are being actively depredated

by GDAWR for the protection of endangered Guam rails and their nests. It is likely that several small Mariana monitors succumbed to acetaminophen toxicosis and would continue to do so if acetaminophen were used as part of a brown treesnake eradication programme on Cocos Island. Small Mariana monitors were also frequently caught live in traps; these could be released unharmed, but GDAWR requested that trapped Mariana monitors be removed and euthanised rather than released, in furtherance of their Guam rail protection efforts. Some small coconut crabs and hermit crabs expired in brown treesnake traps, likely due to dehydration, although the numbers would not be expected to have population-level impacts. Factors such as these need to be considered when assessing the potential environmental impacts of baiting or trapping for invasive snakes, although ineffectiveness in our case could make these issues moot.

The practical information on baiting and trapping is of little avail when brown treesnake removal by these techniques is almost completely ineffectual in the context of abundant alternative prey availability. Initial eradication discussions for Cocos Island included a notional plan of a 20 × 20-m grid of bait stations and a 40 × 40-m grid of traps. At this density, we might have arrayed as many as 825 bait stations and 206 traps with live mouse lures across the entire 33 ha of the Island for the duration of an eradication attempt that is expected to last at least 5 to 10 years (based on ad hoc population estimates and demographic projections; USDA Wildlife Services (2021a)). The pilot studies we report here have forestalled what might have been very costly investments in baiting and trapping with little or no payoff. Financial estimates for the costs of an island-wide baiting and/or trapping effort are beyond the scope of this article, complicated and inflated by considerations, such as boat travel, transect establishment etc., although these issues also affect visual search and removal efforts.

Instead, available funding is being programmed for visual searching and manual removal of snakes, the only tactic that has thus far been effective on Cocos Island. Although night-time visual searching can be logistically challenging, disruptive to work schedules and tedious, it is also the tool that is considered to be the least biased with respect to snake sizes, putting all brown treesnake size classes at risk of detection (Rodda et al. 2007; Christy et al. 2010; Yackel Adams et al. 2018; Lardner et al. 2019b). To date, there have been no documented successful invasive snake eradications beyond a temporary 1-ha experimental exclusion plot (Campbell et al. 2012); however, with judicious and sustained application of the right techniques, the small, isolated island of Cocos Island could potentially be the location of the first successful island-wide eradication.

Further work would be beneficial to validate whether live bird lures would be more effective than mice and carrion on Cocos Island. Field and laboratory experiments have demonstrated that traps with a live bird as the lure show increased capture rates of large, well-conditioned snakes, as well as longer trap investigation times overall compared to those with mouse lures (Yackel Adams et al. 2019; Klug et al. 2021a; Nafus et al. 2021). Additionally, a pattern of feeding success on birds may reduce a brown treesnake's interest in rodents and this may be particularly true on an island where rodents were eradicated in 2009. Verification of the disinterest of Cocos Island snakes to all potential attractant-based lures would be an important next step.

In the event of future invasions, prospects for complete removal of brown treesnakes from larger, prey-rich islands with difficult-to-access terrain would be challenging, particularly when our results indicate that application of the newly developed landscape-scale aerial baiting technology would be ineffective (Siers et al. 2020b). Work to differentiate between the context dependency of control tool attraction, based on total prey availability or species compositions of prey, can also be informative in amending current interdiction programmes or developing emergency response protocols if an incipient population of brown treesnakes is located on another island.

Conclusions

Our results indicate that standard invasive brown treesnake control tools, acetaminophen baiting and trapping with mouse lures, are seemingly not effective enough to warrant significant investment of limited project resources where preferred alternative prey are abundant. These results have profound ramifications for the potential of rapid removal and eradication of incipient brown treesnake populations on any other islands at risk of invasion, such as the Hawaiian Islands, the Northern Mariana Islands and throughout Micronesia and the rest of the Pacific where snake-free islands are rich in diversity and density of potential prey (e.g. Lardner et al. (2019a), Table 1). This work underscores the benefits of a continued emphasis on interdiction – prevention of accidental translocation through strong pre- and post-border inspections and reduction of potential stowaways in high-risk areas on Guam – over reliance on early detection and rapid response to resolve any new brown treesnake invasions that might occur. These results are also important for consideration of prevention, early detection, rapid response, suppression and/or eradication of other invasive snakes on islands or elsewhere. Similar issues are likely to be faced during any invasive snake removal programme, particularly where abundant alternative prey is available.

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Asymmetrical insect invasions between three world regions

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Abstract

The geographical exchange of non-native species can be highly asymmetrical, with some world regions donating or receiving more species than others. Several hypotheses have been proposed to explain such asymmetries, including differences in propagule pressure, source species (invader) pools, environmental features in recipient regions, or biological traits of invaders. We quantified spatiotemporal patterns in the exchange of non-native insects between Europe, North America, and Australasia, and then tested possible explanations for these patterns based on regional trade (import values) and model estimates of invader pool sizes. Europe was the dominant donor of non-native insect species between the three regions, with

most of this asymmetry arising prior to 1950. This could not be explained by differences in import values (1827–2014), nor were there substantial differences in the sizes of modelled invader pools. Based on additional evidence from literature, we propose that patterns of historical plant introductions may explain these asymmetries, but this possibility requires further study.

Keywords

International trade, non-native insects, plants, propagule pressure, species pools

Introduction

Non-native insects have been implicated in displacing native species, altering the composition of ecological communities, damaging economically important trees and food crops, vectoring diseases, and more (Kenis et al. 2009; Bradshaw et al. 2016). An intriguing aspect of insect invasions is that some regions appear to have donated disproportionately more non-native insects during biotic exchange than others. For example, considerably more phytophagous forest insects have invaded North America from Europe than the reverse (Niemelä and Mattson 1996), and Europe has contributed a large fraction of New Zealand's non-native insect fauna (Edney-Browne et al. 2018). Consequently, the question of why such asymmetries may occur has fascinated ecologists for decades, with several mutually compatible hypotheses offered: (1) differences in the magnitude of invasion vectors, such as international trade, may lead to differences in the arrival and establishment rates of non-native species; (2) differences in the size of potential invader pools may drive differences in the numbers of species being donated to other regions; (3) environmental differences (e.g., climate and availability of host plants) in recipient regions may promote or inhibit invasion; and (4) biological traits of insects native to some regions may make them better at invading or competing than insects native to other regions (Vermeij 1991, 1996; Niemelä and Mattson 1996; Visser et al. 2016).

The latter two hypotheses are often tested on a single insect order or guild and at smaller spatial scales (e.g., Rigot et al. 2014; Guyot et al. 2015; Rassati et al. 2016), but less commonly on multiple insect orders and multiple geographical regions. Testing them requires regional knowledge of the nature of recipient environments and their ecological communities, and of the biological traits of the invaders, information that is often available only for certain regions or certain insect groups/guilds. The former two hypotheses are more approachable, given the availability of datasets on international trade, regional insect richness, and modelling approaches that can estimate invader pool sizes.

Our research goals were firstly to test for the existence of asymmetries in the cumulative numbers of insect invaders, across all taxa, exchanged between three world regions of interest: North America, Europe, and Australasia (limited to Australia and New Zealand). These regions were chosen due to their histories of anthropogenic interactions and exchange of species, existing literature suggesting asymmetrical exchange of insects between them (see above), and the availability of data. Secondly, if clear

asymmetries were found, we aimed to determine if they could be explained by differences in propagule pressure (using the value of international trade as a proxy) or by differences in estimates of invader pool sizes. We did not statistically test hypotheses (3) and (4), above, but considered them as possible explanations for asymmetries that could not be explained by hypotheses (1) and (2).

Methods

Datasets and world regions

Insect establishment data were based on the International Non-native Insect Establishment database (Turner et al. 2021), supplemented by several other online datasets (Seebens et al. 2017; Nahrung and Carnegie 2020; Liebhold et al. 2021; GBIF.org 2022; Mally et al. 2022). We used an automated taxonomic cleaning script (Blake and Turner 2021) using the GBIF (GBIF.org 2022) API to standardize species names (merge synonyms and correct misspellings). The resulting dataset contained dated records of non-native insect discoveries, the species identity (order, family, genus, and species), the region/nation in which the species was discovered, the native biogeographic range of the species, and other data such as whether the introduction was deliberate, if the species was found only indoors (e.g., greenhouses), and if the species is herbivorous.

Our choices of world regions and their spatial extents were constrained by the available data. We used a subset of the establishment database that allowed us to compare the reciprocal flows of insects between donor and recipient regions. The only regions that could be compared in this way were North America (NA), Europe (EU), and Australia and New Zealand combined into an Australasian region (AU). Due to spatial gaps in these data, there were minor mismatches in the spatial extents of these regions depending on context. For example, as a donor region, Australasia included Papua New Guinea, but as a recipient region, it only included Australia and New Zealand because we did not have non-native insect discovery records for Papua New Guinea. In this case, correcting for this mismatch would require estimating the number of insects from North America and Europe that have established into Papua New Guinea, and excluding species that also established into Australia or New Zealand. Since the spatial mismatches were relatively minor, and such corrections would themselves be prone to error, we opted not to attempt corrections.

For all analyses, we excluded discovery records where: (1) species had native ranges spanning multiple biogeographic regions (e.g., Holarctic or cosmopolitan species); (2) the native ranges and establishment regions were the same (indicating species that spread within these regions); (3) the establishment was limited to “indoors” (e.g., greenhouses); or (4) the establishment was a result of intentional introduction. This left us with a dataset of 2,324 non-native insect discovery records across the six pairwise flows between North America, Europe, and Australasia, with the dated records spanning 1617–2021.

Regional import value data were obtained from the TradeHist database (Fouquin and Hugot 2016), modified with modern ISO-3 country codes. The TradeHist database describes the annual value of trade goods from 1827–2014 in British pounds sterling (not corrected for inflation) flowing from origin to destination countries. The database does not include details on trade volume/frequency or commodity type. We corrected all trade values for inflation relative to 2020 based on the annual percent change of the UK retail prices index (Office for National Statistics 2021). We grouped the origin and destination countries into the same regions as above (North America, Europe, and Australasia), with some minor unavoidable differences where national borders did not follow biogeographic boundaries. From these groupings of countries, we created a subset of the TradeHist database representing the six pairwise flows between North America, Europe, and Australasia by summing annual trade value across all countries within each region. Records of trade between countries within each of the resulting biogeographic regions were dropped.

Testing for asymmetries and temporal variation in establishment rates

To test for invasional asymmetries, we tallied the number of first discoveries of non-native insects for each of the six pairwise flows between North America, Europe, and Australasia. We further split these cumulative counts by insect order and (separately) by herbivory (herbivores vs non-herbivores). We used G-tests (log-likelihood ratio goodness-of-fit tests) to compare these counts between each donor/recipient pair, separately for each order and herbivory category (e.g., one test for the counts of Coleoptera exchanged in both directions between Europe and North America, another for Hemiptera, etc.), with the null hypothesis being equal numbers of insects exchanged in each direction. We adjusted the *P*-values for multiple comparisons across orders and herbivory categories using the Holm-Bonferroni procedure. To visualize temporal variation in the establishment rates of insects over each flow, we plotted cumulative discoveries versus cumulative import values following Levine and D'Antonio (2003).

Testing for the effects of differential trade and invader pool sizes

To determine if asymmetries in non-native insect establishments between regions could be explained by unequal trade or invader pools, we adapted Poisson process models from Costello et al. (2007) and Morimoto et al. (2019) in which the number of annually established insects flowing from donor to recipient regions were proportional to annual import values and model-fit invader pool sizes. Because the Poisson-process models required dated annual discovery and import values for each observation, this limited our establishment records to the date range of the TradeHist database. This left us with 1,872 dated establishment records (~80% of the establishment dataset) with corresponding import values from 1827–2014.

Our models estimated the lag between establishments and discoveries, predicting the annual establishments necessary to fit to observed discoveries given the lag estimates. This was done to address concerns over records of first discovery being poor

proxies for the actual timing of establishments given the extended lag frequently occurring between establishment and discovery (Costello and Solow 2003). As discovery probability depends in large part on discovery effort, this allows our models to (indirectly) account for overall differences in discovery effort between flows.

To account for the possibility of “saturation” (depletion of invader pools) that might gradually reduce establishment rates, we used AIC-based model selection to choose between models which included or omitted a rate-limiting component based on the observed number of cumulative discoveries compared to a predicted maximum. All models contained an ‘annual establishment rate’ parameter (r) representing the number of non-native insects per billion pounds sterling of imports prior to any depletion of invader pools. If differences in import values fully explained asymmetries in non-native insect establishments, we would expect no significant differences between reciprocal flows in the value of r .

We omitted an intercept term in our models, forcing them to account for all establishments as a function of imports. We modelled the gradual depletion of invader pools as a non-linear rate-limiting factor based on the idea that early invaders are more likely the best or most numerous invaders, leading to a rapid initial decrease in the probability of establishment per unit of propagule pressure (Liebhold et al. 2017). These modifications were necessary to produce good fits to our data – initial attempts to use the same models as in Morimoto et al. (2019) resulted in nonsensical parameter estimations and poor fits in most cases. Our full model was:

$$\begin{aligned}\lambda_t &= rv_t s_t \\ s_t &= \left(1 - \frac{d_{c,t}}{d_{sat}}\right)^2 \\ N_t &\sim \text{Poisson} \left(\delta_t = \sum_{j=1827}^t \lambda_j p_{jt} \right) \\ p_{j,t} &= \pi (1 - \pi)^{t-j},\end{aligned}\tag{1}$$

where:

λ_t is the predicted number of new non-native establishments in year t ,

r is the number of species established per billion pounds sterling (prior to saturation),

v_t is the value of imports (2020 billion pounds sterling) in year t ,

s_t is a rate-limiting factor of interval $[0,1]$ which approaches 0 as the cumulative number of species discoveries approaches a predicted maximum,

$d_{c,t}$ is the (observed) cumulative species discovered by year t ,

d_{sat} is the number of discoveries after which new establishments cease (saturate),

N_t is the actual number of non-native discoveries in year t ,

δ_t is the predicted number of non-native discoveries in year t ,

$p_{j,t}$ is the probability that a species which established in year j will be discovered in year t ,

and π is the annual probability of discovery.

The cumulative sum of discoveries ($d_{c,t}$) was calculated by summing the number of annual discoveries from the first year of records (1827) to year t , inclusive. We used the sum of discoveries instead of establishments for modelling the saturation of species pools because discovery sums could be easily calculated from the original data. The main drawback to this technique was that it slightly complicated the interpretation of the saturation parameter (d_{sat}): rather than being a direct prediction of the invader pool size, it was the predicted number of cumulative discoveries at the time of full depletion of the invader pool.

We fit the models to observed annual discoveries (N_j) for each combination of donor and recipient region, minimizing the maximum likelihood as described by Morimoto et al. (2019):

$$L(r, d_{sat}, \pi) \propto \prod_{j=1827+\tau}^t \exp(-\delta_j) \delta_j^{N_j} \quad (2)$$

where $\tau = 20$ as “preservation years” to prevent fitting the model to species that established prior to 1827 (the first year of discovery records in our database) but were discovered after 1827. Without these “preservation years”, δ_t (the predicted number of discoveries in year t) may be underestimated near the start of the dataset because there will be a lack of prior years of predicted establishments from which to model the lagged discoveries (Morimoto et al. 2019). We also used a reduced model which omitted the depletion of invader pools from Eq. (1), effectively making s_t a constant with a value of 1. We then removed the associated parameter (d_{sat}) from Eq. (2). This “without saturation” model was otherwise identical to the full model.

For parameter estimation, we set lower and (in a few cases) upper bounds on each parameter using the Limited-memory Broyden–Fletcher–Goldfarb–Shanno algorithm (L-BFGS-B) method (Byrd et al. 1995). We bounded the rate of establishments (r) to ≥ 0.005 non-native species per billion pounds sterling of imports. This was done to prevent model optimization from testing ecologically nonsensical parameter estimates (negative or zero species introductions per billion pounds sterling of imports); 0.005 was chosen to be well below the initial slopes of the curves of cumulative non-native species versus cumulative import values. We bounded the annual probability of discovery (π) to between 0.0125 and 0.95 (corresponding to 1.05–80 years of discovery lag), which was chosen as an ecologically reasonable range based on discovery lag estimates from prior publications (Morimoto et al. 2019; MacLachlan et al. 2021). If the lag estimates were left unbounded, the models typically failed to converge. For the saturation term (d_{sat}), we set the lower bound to the cumulative numbers of observed, dated discoveries of non-native insects (since we know the invader pool must be at least this large). This lower bound varied for each of the six flows (1121 for EU to NA, 205 for NA to EU, 349 for EU to AU, 70 for AU to EU, 74 for NA to AU, and 53 for AU to NA). We did not impose an upper bound on the estimates of d_{sat} . We fit both the full and reduced models (the latter lacking the saturation term) to each flow and selected the one with the lowest Akaike information criterion (AIC) value.

To determine if asymmetries may be explained by differences in the size of invader pools, we compared 95% confidence intervals of the predicted numbers of non-native

insect discoveries after full depletion of the invader pool (d_{sat}) between region pairs resulting from our Poisson process models. This was only done when full models (including terms for finite invader pools) were selected for both directions between region pairs. Additionally, we compiled counts of described native insects in each of the three regions for qualitative comparisons to the magnitude of insect invasions across the six flows.

We used the R function *optim* for parameter estimation in the Poisson process models (R Core Team 2021). The confidence intervals were approximately calculated using the inverse of the Hessian matrix evaluated at the last iteration in the optimization process. For parameters with lower or upper bounds, we truncated the confidence intervals to the parameter estimation boundaries. All analyses were performed in R 4.1.0 (R Core Team 2021). Model predictions (cumulative annual establishments and discoveries) were included on the plots of cumulative discoveries versus cumulative trade.

Results

Europe has donated approximately six times more non-native insect species to North America and Australasia than it has received from these regions (Fig. 1).

Asymmetries in the reciprocal flows of non-native insects between Europe and North America and between Europe and Australasia were highly significant in total species, across the five largest insect orders, and among both herbivores and non-herbivores (all $p < 0.001$; Table 1). There were no significant asymmetries in the numbers of non-native insects exchanged between North America and Australasia (all $p > 0.05$; Table 1).

Plots of cumulative insect establishments versus cumulative import values over time show that the European asymmetry developed quickly and early (Fig. 2). In

Table 1. Counts of non-native insect species discovered for each of the six pairwise flows between North America (NA), Europe (EU), and Australasia (AU), by taxonomic order, herbivory, and sum totals. Col. = Coleoptera, Hem. = Hemiptera, Hym. = Hymenoptera, Lep. = Lepidoptera, Dip. = Diptera. The G statistic was computed to test the null hypothesis of no difference in the number of species exchanged in each direction between a given pair of world regions, separately for each column. We used the Holm-Bonferroni method to control for multiple comparisons across orders and herbivory.

Flow	Order						Herbivory		Total
	Col.	Hem.	Hym.	Lep.	Dip.	Other	Yes	No	
EU to NA	477	368	211	144	138	76	854	560	1414
NA to EU	40	72	54	20	29	15	160	70	230
G ($df=1$)	435	218	99.4	106	77.3	44.7	522	434	948
p ($\geq G$)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
EU to AU	137	96	67	31	55	57	226	217	443
AU to EU	34	10	14	7	4	8	41	36	77
G ($df=1$)	66.5	80.7	37.7	16.4	52.5	41.6	141	144	285
p ($\geq G$)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
NA to AU	20	26	13	8	10	11	57	31	88
AU to NA	18	22	15	4	6	7	48	24	72
G ($df=1$)	0.11	0.33	0.14	1.36	1.01	0.90	0.77	0.89	1.60
p ($\geq G$)	- 1	- 1	- 1	- 1	- 1	- 1	0.69	0.69	0.21

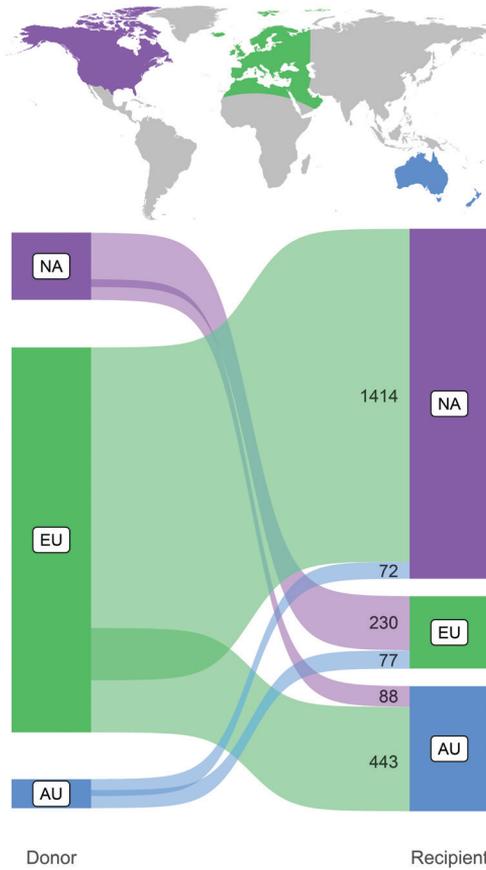


Figure 1. Flows of non-native insects between North America (NA), Europe (EU), and Australasia (AU). Numbers indicate the total count of species established from donor to recipient, with flow widths being proportional to these counts. Overlapping flows on the donor side indicate the fraction of species that established in both recipient regions.

many cases (Europe to North America and Australasia, and North America to Australasia), the rates of establishment of non-native insects per billion pounds sterling (hereafter referred to simply as establishment rates) were greatest near the very start of the dataset (circa 1827). These rates decreased over time, particularly between 1940 and 1960, with our models explaining these declines as depletion of the invader pools. Approximately 75% of non-native insect species that established from Europe into North America and Australasia had done so by 1950 (Fig. 2a, c). In contrast, the establishment rates of North American insects into Europe have decreased only very slightly over time (Fig. 2b), and there is no evidence (as per AIC-based model selection) of any decline in the establishment rates of Australasian insects into Europe (Fig. 2d, Table 2).

Discoveries and modelled establishments of non-native insects between North America and Australasia were within the same order of magnitude in both directions

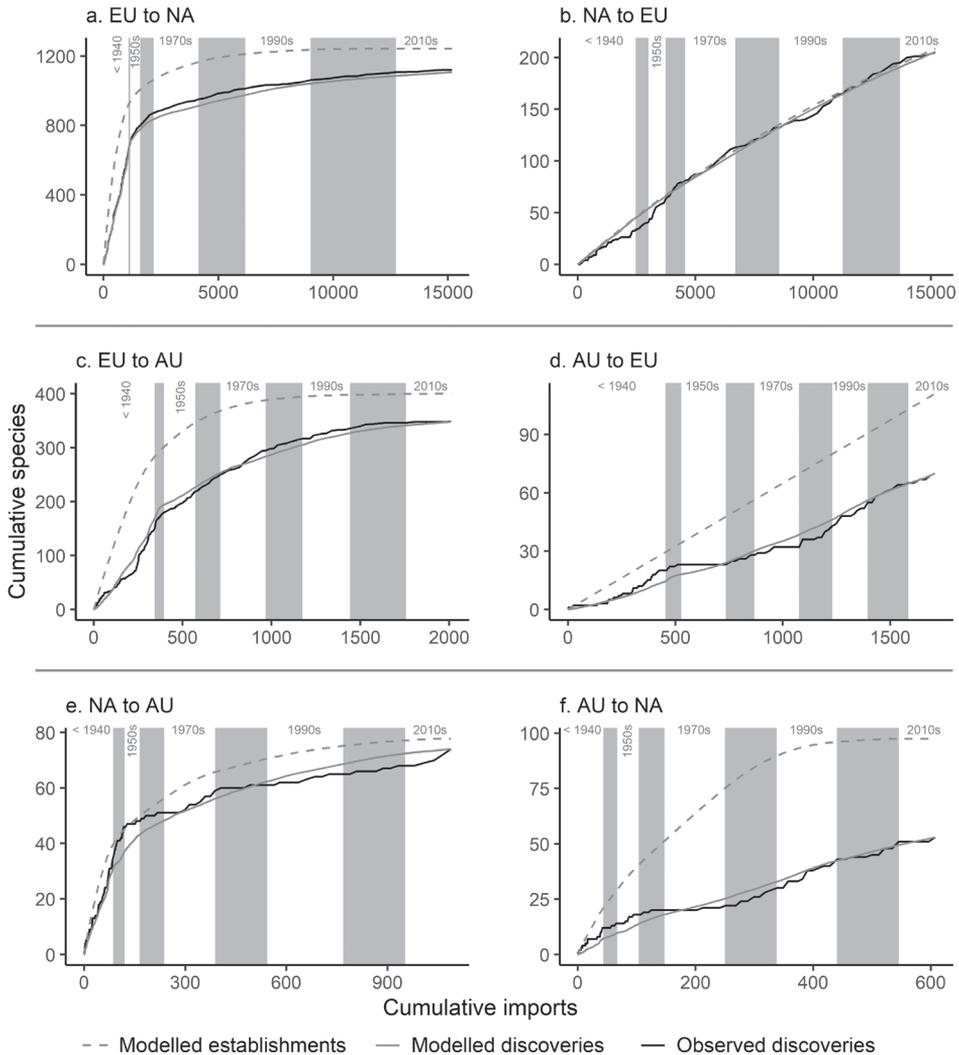


Figure 2. Cumulative discoveries (observed and modelled) and establishments (modelled) of non-native insects exchanged between Europe (EU), North America (NA), and Australasia (AU) versus cumulative import value (inflation-corrected to 2020 British pounds sterling, billions), 1827–2014. Alternating background shading indicates decadal increments, with shading omitted prior to the 1940s for clarity.

(Fig. 2e, f). There was evidence of saturation in the flows of non-native insects between North America and Australasia, though less so from Australasia to North America.

The modelled numbers of non-native insect establishments per billion pounds sterling (annual establishment rate, r) were significantly different for the reciprocal flows between Europe and North America and between Europe to Australasia (Table 2). Between Europe and North America, the predicted sizes of the invader pools (based on the number of discoveries at maximum establishments, d_{sat}) favors Europe, but with overlapping 95% confidence intervals (Table 2).

Table 2. Parameters and 95% confidence intervals of Poisson-process models of establishments and lagged discoveries of non-native insect species exchanged between Europe (EU), North America (NA), and Australasia (AU). All models included a parameter for imports (r , the number of annual establishments per billion pounds sterling) and lag (π , the annual probability of discovery of established species). Models including an additional term for saturation (a decrease in establishment probability as the cumulative number of discoveries approaches d_{sat}) were selected in most cases, with model selection based on Akaike information criterion (AIC) values.

Flow	Best model (Δ AIC of next-best model)	Annual establishment rate, r (95% CI)	Discoveries at maximum establishments, d_{sat} (95% CI)	Annual discovery probability, π (95% CI)	Lag years (95% CI)
EU to NA	Imports + saturation + lag (2118)	1.58 (1.40–1.77)	1121 (1089–1153)	0.0277 (0.0345–0.0208)	36.1 (29.0–48.0)
NA to EU	Imports + saturation + lag (6.08)	0.0194 (0.0144–0.0245)	701 (290–1114)	0.499 (1–0)	2.0 (1.00– ∞)
EU to AU	Imports + saturation + lag (251)	1.212 (0.825–1.60)	366 (312–419)	0.0245 (0.0386–0.0103)	40.9 (25.9–96.7)
AU to EU	Imports + lag (2.0)	0.0647 (0.0173–0.112)	n/a	0.0259 (0.0690–0)	38.5 (14.5– ∞)
NA to AU	Imports + saturation + lag (99.6)	0.771 (0.448–1.09)	76 (68–83)	0.0721 (0.141–0.00354)	13.9 (7.11–283)
AU to NA	Imports + saturation + lag (8.37)	0.621 (0–2.23)	53 (1.60–104)	0.0153 (0.0598–0)	65.5 (16.7– ∞)

* Although this low Δ AIC could be considered “substantial evidence” for both the full and reduced model (Burnham and Anderson 2004), the d_{sat} parameter estimate in the full model greatly exceeded the number of insect species in the donor region, thus the full model effectively lacked saturation and was not ecologically appropriate.

Discussion

Considerably more insect species have invaded North America and Australasia from Europe than in the opposite directions. This concurs with the previously observed overrepresentation of tree-feeding insects from Europe in North America (Niemelä and Mattson 1996), and with non-native insects from the western Palearctic (i.e., Europe) being overrepresented in New Zealand (Edney-Browne et al. 2018). Our results demonstrate that these asymmetries are consistent across all insect orders considered in the analysis, including both herbivorous and non-herbivorous insects.

International trade is considered the single most important pathway for unintentional introductions of insects (Brockerhoff and Liebhold 2017), and greater trade activity generally results in greater propagule pressure of non-native species. Existing literature identifies a positive correlation between import value and the establishment of non-native species (Levine and D’Antonio 2003; Seebens et al. 2017; Lantschner et al. 2020; MacLachlan et al. 2021). Similarly, our models provided excellent fits of inflation-corrected import values to temporal changes in non-native insect establishment rates (after accounting for gradual depletion of source pools). However, the modelled establishment rates (r), which represent the maximum rates of establishments per billion pounds sterling of imports prior to any depletion of source pools, differed significantly between the Europe to North America flow and its converse, and between the Europe to Australasia flow and its converse (Table 2). These significant differences

indicate that even after accounting for differences in trade values, large asymmetries between flows remain unexplained by the models. Contrary to our expectations, and despite the important role of trade in facilitating the establishment of non-native species, we must look to other explanations for these asymmetries.

Temporal variation in establishment rates may hold some clues as to the possible causes of the invasional asymmetries. While global establishments of non-native species have not slowed (Seebens et al. 2017; MacLachlan et al. 2021), our results show that establishment rates may be slowing down at regional scales. Establishments of European insects in North America and Australasia per billion pounds sterling of imports have drastically decreased since 1950 (Fig. 2a, c). Several authors have noted or predicted similar declines in the rate of accumulation of exotic species into the United States (Levine and D'Antonio 2003; Liebhold et al. 2017; MacLachlan et al. 2021; Seebens et al. 2021), with two possible explanations offered: depletion of source invader pools, or improved biosecurity measures.

Unequal flows of non-native insects may arise from differences in the numbers of potential invaders present in the donor regions (Vermeij 1991). Our models attempted to predict the size of these invader pools, provided that a decreasing trend in establishment rates could be suitably explained (based on ΔAIC) by the depletion of these pools. Our results suggest that some of the asymmetry in non-native insects exchanged between Europe and North America may be due to a ~60% larger pool of European insect invaders. However, this was not a significant difference, given the large confidence intervals around these estimates. Described insect species richness in Europe is approximately equal to that of North America (de Jong et al. 2014; Arnett 2000). Again, this suggests that the asymmetry between Europe and North America cannot be explained by differences in invader source pool sizes (assuming that the ratio of described to undescribed species is not strikingly greater in North America and Australasia than it is in Europe).

Scientific effort almost certainly varies regionally, and this may impact the interpretation of our results. Over the last few hundred years, Europe has had a consistently greater population density than either North America or Australasia (Goldewijk 2005). If this corresponds to greater scientific effort in Europe (more biologists/naturalists per unit area), the proportion of established species which have been discovered and the proportion of native species which have been described are likely greater in Europe than in North America and Australasia. This has several implications for our analyses. First, it suggests that we may have relatively underestimated the numbers of European insects in North America and Europe and thus the asymmetries may be even more dramatic than our analyses suggest. Second, the published counts of described insect species may be biased in favor of a relatively greater number in Europe, which may predispose us towards suggesting that the asymmetries are due to a greater richness of European invaders. We attempted to account for differential scientific effort in our models, in the form of an annual probability of detection that could vary independently for each of the six flows. Unfortunately, this parameter seldom had a clear optimum, and the resulting confidence intervals are large. These wide confidence intervals may be due in part to using a fixed annual probability of detection (unchanging over

time). Future research may benefit from allowing the discovery probability to change over time, perhaps by incorporating regional time series of proxies for scientific effort.

Despite the lack of statistical significance, the larger estimate for the pool of European insect invaders in North America versus the opposite could be considered a point in favor of the European crucible hypothesis proposed by Niemelä and Mattson (1996). This hypothesis suggests that a history of extensive glaciations may have reduced the niche diversity and ‘invasibility’ of Europe by leading to extinctions of plant genera, while simultaneously selecting for competition-hardened species that thrive in disturbed habitats, making European species better invaders. However, Europe has been heavily colonized by insects from regions other than North America, particularly the Asian Palearctic (Roques et al. 2020), suggesting that Europe is not notably resistant to invasion. Additionally, our models suggest that European insects established into North America 81 times more quickly (per billion pounds sterling of imports) than North American insects established into Europe. If this remarkable difference could be explained largely by European insects being better invaders, we would expect model estimates of the invader pool sizes between Europe and North America (using d_{sat} as a proxy) to be considerably more different than they were found to be, and significantly so.

Although we have modelled declining establishment rates as the gradual depletion of source invader pools, it is also likely that biosecurity measures have contributed. International biosecurity regulations, specifically phytosanitary measures, began in earnest in the 20th century (Roques 2010; Allen et al. 2017). With plant-feeding insects making up 58% of all non-native insect species in our dataset, strengthened phytosanitary measures applied to pathways including live plants, wood, and crops have almost certainly led to contemporary reductions in establishment rates. Europe has also had less strict phytosanitary measures than Australia and New Zealand for many decades (Eschen et al. 2015), which may partly explain the relative lack of declines in the rates of discoveries of North American and Australasia insects (per billion pounds sterling) in Europe. However, strengthening biosecurity efforts are most likely to have influenced the latter half of our time series (1900 and onwards), whereas considerable asymmetry in the numbers of species exchanged between Europe, North America, and Australasia had already accumulated by 1900. Therefore, differential biosecurity is an unlikely driver of these asymmetries.

Though historical invasion discoveries began much earlier, available import data only began in 1827. Given that the greatest establishment rates were seen at the very start of the dataset, it is possible – perhaps even likely – that the main causal agents explaining the dominance of Europe as a source of non-native insects in North America and Australasia were transient phenomena that began prior to 1827. This is complicated further by invasion biology being a relatively new discipline: early records of novel species may be both lacking and underrepresented in scientific literature. After a non-native species establishes, there is typically a time lag until it is discovered (Essl et al. 2011). Although our models attempt to account for lags between establishment and discovery, we used an annual probability of detection that does not change over time, and the lag estimates often have wide confidence intervals. Therefore, we are not confident that our data could be used to extrapolate far into the past.

Well before our dataset begins, North America and Australasia were experiencing a period of dramatic change as European colonies were founded. This colonization promoted both deliberate and accidental introductions of European plants (Lenzner et al. 2018). Introductions of exotic plants by colonial powers accelerated in the 19th and early 20th centuries, with a lasting impact on the global composition of floral communities (Lenzner et al. 2022). This is noteworthy, because plant imports and introductions may be a strong predictor of insect invasions (Liebhold et al. 2012; Liebhold et al. 2018; Bonnamour et al. 2023). North America and Australasia each have nearly twice as many extra-continental non-native plant species as Europe, and Europe is second to Asia as a dominant source of non-native plants worldwide (van Kleunen et al. 2015). This history, and the close relationships between insects and plants, suggest a potential explanation for both the existence of the asymmetries we observe and their temporal trends. We suggest that future research focuses on European colonization and coincident plant introductions as possible explanations for why Europe has donated so many more non-native insects into North America and Australasia than it has received from these regions.

We cannot rule out other factors not addressed here, such as differences in establishment probability driven by climate suitability or biotic resistance, the effect of establishments originating from non-native populations ('bridgeheads'), or differences in propagule pressure driven by the specific types of trade goods exchanged between regions. This latter factor is likely the most important to consider for future research, as overall import values may not capture changes over time in the relative contribution of specific commodities (such as plants and plant products) to overall trade. From the discussion above, we know to expect close associations between insects and plant products. Plant products may also have low values per unit of volume, thus being poorly represented in overall import values. Analyses which considered different commodities separately were conducted by Morimoto et al. (2019) but these were limited to Japan, 1951–2016. Data for continent-scale regions (i.e., North America and Europe) going back into the early 1800s do not exist, so far as we are aware, in any cohesive form. To compile such data from historical records would be a major interdisciplinary effort and was beyond the scope of our present research. Regardless, our results are an important step forward in confirming the existence of strong asymmetries in insect establishments between our focal regions and suggesting possible explanations for their cause.

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Supplementary material I

Data for Asymmetrical insect invasions between three world regions

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Data type: docx

Explanation note: Annual (1827–2014) and undated discoveries of non-native insects and annual inflation-corrected import values (in 2020 British pounds sterling, billions) exchanged between Europe (EU), North America (NA, north of Mexico) and Australasia (AU, Australia and New Zealand only).

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Worldwide distribution and phylogeography of the agave weevil *Scyphophorus acupunctatus* (Coleoptera, Dryophthoridae): the rise of an overlooked invasion

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Abstract

Global plant trade represents one of the main pathways of introduction for invertebrates, including insects, throughout the world. Non-native insects include some of the most important pests affecting cultivated and ornamental plants worldwide. Defining the origins and updating the distribution of non-native invasive species is pivotal to develop effective strategies to limit their spread. The agave weevil, *Scyphophorus acupunctatus* (Coleoptera, Dryophthoridae), is a curculionid beetle native to Central and North America, although it also occurs in Eurasia, Africa, Oceania and South America as a non-native species. Despite being widespread, the extent of occurrence and origins of European populations of the agave weevil have been overlooked. In the present study, the current and potential worldwide distribution of *S. acupunctatus* was assessed and an analysis of its genetic diversity in the native and non-native ranges was performed. By analysing occurrences from local phytosanitary bulletins and citizen-science platforms, the agave weevil was confirmed to be widely distributed and to occur on all continents, except Antarctica. Additionally, there is potential for expansion throughout the world, as estimated by species distribution models. Nucleotide and haplotype diversity of the COXI mitochondrial gene (about 650 bp) was lower in the non-native ($n = 39$ samples) than native populations ($n = 26$ samples). The majority of introduced

individuals belonged to the same haplotype, suggesting that most introductions in Europe might have occurred from a small geographical area in Central America. Constant transboundary monitoring and national laws must be considered to reduce the spread of the agave weevil, given that a bridgehead effect may occur from non-native populations to new suitable areas.

Keywords

Agave, mitochondrial COXI gene, non-native invasive insects, population genetics, species distribution model

Introduction

Non-native invasive species are taxa that have been introduced and/or spread into regions outside their native ranges and have subsequently established and spread, affecting local ecosystem dynamics (CBD 2010). Since the Holocene and the earliest explorers, human migration has been essential to the movement of species from their native ranges to areas where they were not present (Foster et al. 2002; Banks et al. 2015). Globalisation has intensified the human-assisted spread of living species in non-native areas, following international trade and human journeys (Meyerson and Mooney 2007). In addition, the creation of ecological corridors has facilitated the range expansion of many taxa in non-native countries (Mattson et al. 2007; Horsák et al. 2019).

Crop pests are widely distributed worldwide due to accidental introductions through the intensive trade of goods, including plants of ornamental and agronomic interest (Deutsch et al. 2018). Amongst crop pests, many invertebrate species have been thoroughly studied, particularly in biocontrol and pest management research (Geier 1966; Parsons et al. 2020). Well-studied crop pest species include *Halymorpha halys* (Stål) and *Tuta absoluta* (Meyrick) (Biondi et al. 2018; Cianferoni et al. 2018). However, most non-native insect pests have been poorly investigated and their impact and distribution are currently still under assessment (e.g. *Corythauma ayyari* (Drake) and *Stator limbatus* (Horn): Mazza et al. (2020); Cocco et al. (2021)). For instance, palms in Mediterranean countries are threatened by both the well-known red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Soroker and Colazza 2017) and the still mostly unknown and overlooked palm borer moth, *Paysandisia archon* (Burmeister) (Mori et al. 2023). Curculionid coleopterans (e.g. weevils) are an important threat to many cultivated species including corn, figs, palms and other ornamental plants (Guzmán et al. 2012; Inghilesi et al. 2015; Farina et al. 2020). Amongst those, the agave weevil, *Scyphophorus acupunctatus* Gyllenhal (Coleoptera, Dryophthoridae), is one of the least-studied species. This weevil is native to southern North America, Mexico and other countries in Central America (Vaurie 1971), although it has been introduced to several parts of the world including American islands and South America (US Virgin Islands and Hawaii, Cayman Islands, Puerto Rico, Cuba, Haiti, Jamaica, Dominican Republic, Curaçao, Colombia, Venezuela and Brazil), Africa (Kenya, Tanzania and South Africa), Asia (Indonesia and Saudi Arabia), Oceania (South Australia and Fiji Islands) and Europe (Portugal including Madeira, Spain including Canary and Balearic

Islands, France, Italy, Croatia, Greece and Cyprus: Setliff and Anderson (2011); CABI/EPP0 (2014); Vassiliou and Kitsis (2015); Andrade (2022); Pernek and Cvetković (2022)). Populations of *S. acupunctatus* in Central America (Honduras, Belize, Guatemala, Costa Rica, El Salvador and Nicaragua) have an uncertain origin, as it is unclear whether they are native or not. These populations may represent an undocumented natural range expansion from northern countries, i.e. Mexico, in recent times or they might have been introduced through plant and horticultural trade (Vassiliou and Kitsis 2015; EPP0 2022a). Occurrences of the agave weevil in Israel, New Zealand, Queensland (Australia), Argentina and the United Kingdom that have been reported by some authors (CABI 2023), have never been confirmed in the scientific literature, nor in citizen-science platforms or social networks. In general, the distribution of this weevil is mainly known at the country level, with little known about its actual distribution within each country (Martín-Taboada et al. 2019).

The agave weevil is a major pest of agave. Agaves (Asparagaceae, Agavoideae/Agavaceae) include several genera and species that have been introduced worldwide for ornamental purposes (Thiede et al. 2019). Most agave species are susceptible to this weevil, particularly those belonging to the genus *Agave* (Vaurie 1971; Bolaños et al. 2014; Palemón-Alberto et al. 2022). Plants are directly damaged by the agave weevil whose larvae feed on agave heads by boring galleries (Figueroa-Castro et al. 2016). The consumption of plant parts by the agave weevil larva may cause plant mortality (Aquino-Bolaños et al. 2013). Adults cause little damage in comparison to larvae.

The taxonomy of the *Scyphophorus* genus is still unresolved (Chamorro et al. 2016). Although two species are traditionally recognised, *S. acupunctatus* and *S. yuccae* Horn, no reliable information on the phylogeography of this genus is available. Genetic analyses of *Scyphophorus* spp. have been carried out on a limited sample size or in limited geographical areas of Central America (Azura-Domínguez et al. 2013; Chamorro et al. 2016). Furthermore, no molecular data are available to disentangle the two *Scyphophorus* species, given that the only deposited sequence of *S. yuccae* is actually belonging to *S. acupunctatus*, questioning the actual validity of the former species (Chamorro et al. 2016). Assessing the geographic origin of non-native populations of *S. acupunctatus* may help to provide information for plant trade controls and assist with preventing new invasions. Although *S. acupunctatus* is also recorded in Africa, SE Asia and Australia, most non-native populations of this weevil occur in Europe, where *S. acupunctatus* has been introduced through the plant trade (e.g. *Beaucarnea recurvata* Lem., *Agave americana* L. and *Yucca* spp.), most likely from different countries of Central and North America (e.g. from Nicaragua to Italy: EPP0 (2022b)). The agave weevil was reported for the first time in the Netherlands in 1980 (van Rossem et al. 1981) and, subsequently, in Italy, France, Spain and Greece (Colombo 2000; Flinch and Alonso-Zarazaga 2007; EPP0 2008; Kontodimas and Kallinikou 2010). Therefore, we focused mostly on European and Mediterranean countries, as these countries include most of the non-native range of this weevil species.

The aims of our work were to: (i) update the distribution of the agave weevil in non-native areas with special regard to Mediterranean countries; (ii) determine the climatic

suitability throughout the world, with special regard to Europe, where most non-native populations occur and predict its potential distribution; and (iii) assess the phylogeographic pattern of *S. acupunctatus* and trace the origin of European populations.

Materials and methods

Updating the distribution of the agave weevil

The distribution of the agave weevil in its non-native range was updated by searching for published and unpublished records in the grey and scientific literature and online databases, including records collected through citizen-science and validated by experts (i.e. iNaturalist: www.inaturalist.org; GBIF: www.gbif.org DOI: <https://doi.org/10.15468/dl.pd22mh>; Forum Natura Mediterraneo: www.naturamediterraneo.com; Forum Entomologi Italiani: www.entomologiitaliani.net. All accessed on 15.05.2023). The search for occurrence records was conducted from October 2022 to May 2023. Further searches were performed on free posts with photos on Social Networks (e.g. Facebook) and on video-sharing websites (e.g. YouTube). The literature search was carried out by assessing studies in online databases (i.e. ISI Web of Science, Scopus, Zoological Records and Google Scholar). Search terms included all possible combinations of the words: ‘agave weevil’, ‘*Scyphophorus acupunctatus*’, ‘distribution’ and ‘non-native species’. The same words were searched in English, French, Spanish, Portuguese and Italian. Maps representing the agave weevil distribution using geographical coordinates were downloaded from the ESRI (<https://server.arcgisonline.com>) and Eurostat (Countries – GISCO – Eurostat, europa.eu) websites. The distribution of the weevil was mapped using QGIS software version 3.28 Firenze (QGIS Development Team 2019).

The suitability of current and future climates for the agave weevil: preliminary analyses

The potential worldwide distribution of *S. acupunctatus* was modelled to identify areas throughout the Globe that are climatically suitable for this weevil. To the best of our knowledge, no previous studies have focused on the climatic preferences of this weevil, despite its high impact on agro-economy and urban parks.

Occurrence records from both the native and non-native ranges were collected, representing the whole realised ecological niche (Srivastava et al. 2021). This approach resulted in a total of 1525 high-accuracy occurrence records (uncertainty < 1 km). The raw dataset underwent a meticulous analysis to identify and eliminate duplicate entries. This process was carried out in two steps: an initial manual inspection employing the duplicate search function in Microsoft Excel (Microsoft Office 365), followed by subsequent verification using the “duplicated” function of “spocc” package (Chamberlain et al. 2017) in the R software version 4.1.2 (R Core Team 2019). By implementing these measures, overlapping data points from various sources were successfully identified and removed. A final new dataset of 1135 occurrences without duplicates was obtained.

Moran's correlograms were employed to test for the presence of significant spatial autocorrelation (De Marco et al. 2008), using spatial analysis tools available in ArcGIS Pro (ESRI 2011). The spatial autocorrelation analysis was conducted using the final dataset as the input file. In detail, we assessed the spatial autocorrelation between 1 and 10 km at 1 km intervals (De Marco et al. 2008; Crase et al. 2014).

The Moran's correlogram is a graphical representation of the spatial autocorrelation coefficient (Moran's I) at different distance intervals, which helps to identify patterns of spatial dependence and assess whether neighbouring observations are more similar or dissimilar from each other than expected by chance (Crase et al. 2014). The Moran's I coefficient ranges from -1 to 1, where positive values indicate positive spatial autocorrelation (similar values tend to cluster together), negative values indicate negative spatial autocorrelation (dissimilar values tend to be clustered) and values close to zero indicate no spatial autocorrelation (values are randomly distributed across space: Crase et al. (2014), Suppl. material 1: fig. S1).

In this work, the computed Moran's Index was 0.03, indicating a slight positive spatial autocorrelation in the dataset. The Z-score, which measures the standard deviation from the expected mean under the assumption of spatial randomness, was 0.18. The associated P-value was 0.86, suggesting that the observed spatial pattern was not significantly different from what would be expected by chance. Overall, these findings suggested the absence of significant spatial clustering or dispersion in the analysed spatial context. The final dataset used in the model consisted of 718 occurrences.

A distance threshold of 10 km was set to define spatial relationships between observations. This threshold represents the maximum distance at which observations are spatially related. The analysis was performed without any specific selection set, meaning that all observations within the study area were included in the analysis. No weight matrix file was used, suggesting that all observations were assumed to have equal influence in the analysis.

Dispersal abilities of *Scyphophorus* weevils are limited (< 50 metres), as reported by the scientific literature (Huxman et al. 1997; Figueroa-Castro et al. 2016). In line with the spatial autocorrelation analysis, a 10 km distance was selected to filter the occurrences (Di Cola et al. 2017; Montalva et al. 2017; Atauchi et al. 2018; Guevara et al. 2018).

In the final analysis, occurrences were filtered by selecting the minimum distance of 10 km between different occurrence points using the "spThin" R package (Aiello-Lammens et al. 2015). This distance threshold allows for the consideration of occurrences as independent from one another and aligns with the resolution of climate data (Ancillotto et al. 2023).

Selection of variables

The modelling process was started by obtaining 19 climatic variable layers from the Worldclim (version 2.1) website, with a resolution of 2.5 minutes of a degree (Fick and Hijmans 2017). Subsequently, a Principal Component Analysis (PCA: Suppl. material 1: figs S2, S3) was performed using the "ade4" package in R to identify variables with a high collinearity and explore their correlation structure (Fourcade et al. 2014).

Variables were carefully chosen for modelling *S. acupunctatus* by excluding those showing strong intercorrelation. As a result, six highly-significant variables were selected to model the distribution of *S. acupunctatus* (Suppl. material 1: table S1). These variables included BIO1 (Annual Mean Temperature), BIO4 (Temperature Seasonality), BIO6 (Minimum Temperature of the Coldest Month), BIO7 (Temperature Annual Range), BIO9 (Mean Temperature of the Driest Quarter) and BIO11 (Mean Temperature of the Coldest Quarter).

Additionally, the Variance Inflation Factor (VIF) for all selected variables was computed using the “usdm” package in R (Naimi et al. 2014). The VIF values were examined to ensure that all values were below 3, indicating a very low level of multicollinearity (Prakash 2019). Specifically, variables with a Pearson’s correlation coefficient of below 0.70 or above -0.70 were retained (Alin 2010; Kock and Lynn 2012; Regos et al. 2020) (Suppl. material 1: table S2). The six bioclimatic variables that were selected to model the distribution of *S. acupunctatus* under current climatic conditions were also chosen to model the distribution of the species under future climates, spanning from 2041 to 2070. Future climate data were downloaded under the Representative Concentration Pathways (RCP 2.6) scenario. The RCP 2.6 future bioclimatic raster is widely acknowledged in literature as a representative case for mitigation strategies aimed at constraining the rise of global mean temperature to 2 °C (van Vuuren et al. 2011).

Algorithm selection

A first comprehensive evaluation was conducted to estimate the performance of nine algorithms through a combination of R packages such as “ENMeval” and “sdm” (Kass et al. 2021; Montoya-Jiménez et al. 2022).

The evaluation encompassed a range of algorithms, namely the Generalised Linear Model (GLM, with a logit-link function), Boosted Regression Trees (BRT, with 15% holdout validation point and bagging fraction set to 0.5: Mui (2015)), Random Forest (RF, with max. tree depth = 2–4: Valavi et al. (2021)), Maximum Entropy (MaxEnt), Generalised Additive Model (GAM), Multivariate Adaptive Regression Splines (MARS), Geometric Brownian Motion (GBM), BIOCLIM and Functional Data Analysis (FDA: Pecchi et al. 2019; Steen et al. 2021). The goal was to identify the most suitable models for the study and reduce computational efforts. To achieve this, along with presence records, 6000 random background points (1000 background points per continental area where the occurrence of *S. acupunctatus* is reported, i.e. North America, South America, Europe, Asia, Africa and Oceania) were generated (Barber et al. 2022; Buonincontri et al. 2023). In particular, background points were selected in a buffer of 10,000 metres around occurrences, in line with previous literature (Iturbide et al. 2015; Rotllan-Puig and Traveset 2021). Evaluation metrics, such as the Area Under the Curve (AUC) and True Skill Statistics (TSS), were employed to assess the model performance (Suppl. material 1: table S3: Steen et al. (2021)). Unsuitable models (AUC < 0.90; TSS < 0.75) were discarded.

Modelling

Species Distribution Models (SDM) were performed using the R packages “biomod2” and “sdm” (Thuiller 2014; Naimi and Araújo 2016). Following the previous evaluation, only the most suitable model algorithms were selected for the inclusion in the ensemble model. The selection process aimed at choosing models with the highest performance to promote accurate forecasts and ensure reliable results (Thuiller 2014). An ensemble species distribution model was fitted using four algorithms: MaxEnt, RF, GLM and GAM (Araujo and New 2007). By incorporating both statistical and machine-learning approaches, the ensemble approach enables a comprehensive analysis and assessment of the species’ potential distribution, which cannot be reached with a single-model approach when the performance of the individual models is low (Araújo and New 2007; Buisson et al. 2010; Hao et al. 2019). This integration of different modelling techniques enhances the robustness of the analysis and improves the overall understanding of the studied phenomenon. Amongst the obtained models, the RF performed the best, with an AUC = 0.99 and TSS = 0.90 (Suppl. material 1: table S3).

The results of the models were assembled with a weighted average of all predictions from all fitted models (Buisson et al. 2010; Smith et al. 2017). The variables for future projections (2041–2070) were then downloaded. Future projections of these variables were obtained for the emission-conservative scenario known as RPC 2.6. Built models were then projected under future climatic conditions. The bioclimatic rasters for future climates at a 2.5-minute degree resolution were evaluated following the same procedures described earlier (Ancillotto et al. 2016, 2020; Cancellario et al. 2023). This approach provided valuable insights into the possible impacts of climate change on the climatic suitability of the world for the agave weevil. For the RCP 2.6 scenario and for each variable, the median of five Global Circulation Models (GCMs) was used: GFDL-ESM4, UKESM1-0LL, MPIESM1-2-HR, IPSLCM6A-LR and MRI-ESM2-0 (Mori et al. 2023). Models were validated using spatial cross validation with the R package “blockCV” (Valavi et al. 2019). The K-fold cross validation was performed, with K = 5 as determined through the “buffer evaluation”, i.e. by using the function “cv_buffer” (Pohjankukka et al. 2017).

Model performance was measured using TSS and AUC. For present and future projections, an occurrence probability raster was obtained for each statistical model by calculating the mean of all the projections with a TSS > 0.75 and an AUC > 0.90 (Mori et al. 2023).

Then, differences between predictions under future and current climates were obtained using consensus models, by subtracting the average predictions under current climates from those under future climate. Raster cells with positive values indicated a predicted improvement in climatic conditions for *S. acupunctatus*, whereas raster cells with negative values indicated a decreased climatic suitability for the future. To estimate the uncertainty in the predictions due to disagreements amongst four different algorithms, subtraction per model was performed and the following values were assigned: value -1 was assigned to all cells with negative values of the average single-

model predictions; similarly, the value +1 was assigned to all cells with positive values and 0 otherwise (Mori et al. 2023).

The consensus of model predictions was obtained by summing the four three-value maps (-1, 0, 1). A raster map was obtained with values ranging between -4 and +4, with extreme values suggesting that all the four statistical models predicted a decrease (-4) or an increase (+4) in the probability of occurrence, whereas intermediate values indicated a partial (± 2 ; ± 3) or high disagreement (-1 to +1) amongst the predictions of the algorithms (Suppl. material 1: fig. S4).

The potential non-analogue climate was checked using a Multivariate Environmental Similarity Surface (MESS) analysis (Elith et al. 2011; Fischer et al. 2011). The MESS analysis estimates the similarity between environments used to train the model and the new projected areas for every grid cell (Elith et al. 2011). The analysis was used to detect regions with environments that are outside the range of environments in the training area (Fischer et al. 2011). Climatic similarities between regions and periods were determined by MESS values. Negative values represent non-analogue climatic conditions.

Phylogenetic and genetic diversity analysis

A total of 32 individual samples of *S. acupunctatus* were collected in Europe and preserved in 95% ethanol at -20 °C, before genetic analyses. Four other samples from Liguria (Pallanca and Hanbury Botanical Gardens, located in Bordighera and Ventimiglia, respectively, Imperia Province, NW Italy) were previously collected by the CNR-IRET researchers and stored in absolute ethanol at the laboratory of CNR-IRET in Sesto Fiorentino (Florence, Italy) (Table 1).

Genomic DNA from all samples was extracted using QIAGEN Blood and Tissue kit (Qiagen Inc., USA), following the manufacturer's protocol. A fragment of the mitochondrial DNA Cytochrome Oxidase I (COXI) was amplified and compared with sequences deposited in the GenBank. COXI was amplified using the primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198: '5-TAAACTTTCAGGGTGACCAAAAAATCA-3' (Folmer et al. 1994). These primers were previously used to amplify the same gene in *S. acupunctatus* from Central America for species-identification purposes (Azua-Domínguez et al. 2013; Chamorro et al. 2016: Table 2) using the amplification protocol reported by Baratti et al. (2005) and Chamorro et al. (2016).

PCR products were run on a 1.5% agarose gel, then purified (ExoSAP-IT, Amersham Biosciences) and finally sent to BMR Genomics (Padua, Italy) for Sanger sequencing. Electropherograms were visualised with the software Chromas 1.45 (<http://www.technelysium.com.au>. Accessed on 17.12.2022). The sequences were visually corrected and aligned using ClustalX 2.1 (Thompson et al. 1997), together with all the available COXI sequences of *S. acupunctatus* retrieved from GenBank and BOLD System, for a total of 65 sequences (627–903 bp: Table 2).

The phylogenetic reconstruction was conducted by applying Neighbour Joining (NJ), Bayesian Inference (BI) and Maximum Likelihood (ML) methods.

Table 1. Location of the 32 sampling sites for *Scyphophorus acupunctatus* in Europe. Coordinates are expressed in UTM WGS84.

Sample ID	Location of origin	Country	Latitude (°N) / Longitude (°E)
S1	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.04893°N, 8.93734°E
S2	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.04588°N, 8.93496°E
S3	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.04454°N, 8.93399°E
S4	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.04150°N, 8.92494°E
S5	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.03500°N, 8.92161°E
S6	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.03612°N, 8.92197°E
S7	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.03348°N, 8.91776°E
S8	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02657°N, 8.89292°E
S9	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02547°N, 8.89186°E
S10	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02617°N, 8.89052°E
S22	Villamaniscle coll del Quirc, Girona	Spain	42.38092°N, 3.07618°E
S23	Villamaniscle coll del Quirc, Girona	Spain	42.38092°N, 3.07618°E
S29	Tamaracciu, Corsica	France	41.55294°N, 9.31810°E
S30	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02632°N, 8.88836°E
S31	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02580°N, 8.88484°E
S32	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02669°N, 8.88217°E
S33	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.02668°N, 8.88250°E
S34	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.01517°N, 8.88777°E
S35	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.01586°N, 8.88914°E
S36	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.01103°N, 8.88029°E
S37	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.01449°N, 8.87612°E
S38	Isola Rossa - Costa Paradiso, Sardinia	Italy	41.05372°N, 8.94518°E
S44	La Crau, Var	France	43.16317°N, 6.09292°E
S47	Sperlonga, Latium	Italy	41.25847°N, 13.43976°E
S57	Cittadella Universitaria, Catania, Sicily	Italy	37.52546°N, 15.07199°E
S59	Cittadella Universitaria, Catania, Sicily	Italy	37.52546°N, 15.07199°E
S61	Località Balzi Rossi, Ventimiglia, Liguria	Italy	43.78361°N, 7.53638°E
Spal1	Pallanca Garden, Bordighera, Liguria	Italy	43.78835°N, 7.68749°E
Spal2	Pallanca Garden, Bordighera, Liguria	Italy	43.78839°N, 7.68736°E
Shan1	Hanbury Garden, Ventimiglia, Liguria	Italy	43.78408°N, 7.55429°E
Shan2	Hanbury Garden, Ventimiglia, Liguria	Italy	43.78445°N, 7.55415°E
Españ1	Passeig Maritim de la Barceloneta, Barcelona	Spain	41.38474°N, 2.19592°E

The Kimura-2-parameters nucleotide substitution model was selected by jModelTest 2 (Darriba et al. 2012) with the Akaike Information Criterion (AIC) and corrected for rate heterogeneity amongst sites with a Gamma distribution. The NJ was performed by MEGA 11 software with 10,000 bootstrap replicates (Tamura et al. 2021). The BI analysis was performed with MrBayes v.3.12 (Ronquist and Huelsenbeck 2003), using the best model selected. Four chains of Markov Chain Monte Carlo were simultaneously run and sampled every 1000 generations for 4 million generations. The first 1000 sampled trees from each run were discarded as burn-in. The ML phylogenetic analysis was conducted with SeaView software (Gouy et al. 2010). Outgroups (*Dryophthorus corticalis* (Paykull), *Stromboscerini* sp. and *Aclees taiwanensis* Kôno) were selected in line with their close phylogenetic placement within the family to the study taxon. Nucleotide diversity, haplotype diversity, number of parsimony-informative and variable sites were

Table 2. Accession numbers of sequences used for the phylogenetic reconstructions of *Scyphophorus acupunctatus*.

Accession number	Sampling location	Sampling country	Population status	Reference
AY131110	Not available	Continental USA	Native	Direct submission to GenBank
AY131122	Massachusetts	Continental USA	Native	Direct submission to GenBank
GBCL49633-19	California	Continental USA	Native	Direct submission to BOLD Systems
HM433616	Colorado	Continental USA	Native	Direct submission to GenBank
KU896920	Arizona	Continental USA	Native	Chamorro et al. (2016)
KU896921	Arizona	Continental USA	Native	Chamorro et al. (2016)
KU896922	Arizona	Continental USA	Native	Chamorro et al. (2016)
KU896923	Arizona	Continental USA	Native	Chamorro et al. (2016)
KU896924	Arizona	Continental USA	Native	Chamorro et al. (2016)
JX134898	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134899	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134900	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134901	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134902	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134903	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134904	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134905	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134906	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134907	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134908	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134909	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
JX134910	Not available	Mexico	Native	Azuara-Dominguez et al. (2013)
ASSCR6360-12	Not available	Costa Rica	Most likely native	Direct submission to BOLD Systems
ASSCR6362-12	Not available	Costa Rica	Most likely native	Direct submission to BOLD Systems
KU896927	Not available	Guatemala	Most likely native	Chamorro et al. (2016)
KU896929	Not available	Guatemala	Most likely native	Chamorro et al. (2016)
OQ198464	La Crau	Continental France	Non-native	Present work
OQ198455	Corsica	France	Non-native	Present work
OQ193159	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ193160	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ193161	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ193162	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ193165	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ193176	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ193177	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194007	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194008	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work

Accession number	Sampling location	Sampling country	Population status	Reference
OQ194015	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194016	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ198466	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194025	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194031	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194033	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ198456	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ198458	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ198459	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ198460	Isola Rossa – Costa Paradiso, Sardinia	Italy	Non-native	Present work
OQ194017	Balzi Rossi, Ventimiglia, Liguria	Italy	Non-native	Present work
OQ198461	Pallanca Gardens, Liguria	Italy	Non-native	Present work
OQ198457	Pallanca Gardens, Liguria	Italy	Non-native	Present work
OQ193174	Hanbury Gardens, Liguria	Italy	Non-native	Present work
OQ198462	Hanbury Gardens, Liguria	Italy	Non-native	Present work
OQ194018	Catania, Sicily	Italy	Non-native	Present work
OQ194019	Catania, Sicily	Italy	Non-native	Present work
OQ198463	Sperlonga, Latium	Italy	Non-native	Present work
OQ193157	Villamaniscicle	Spain	Non-native	Present work
OQ193158	Villamaniscicle	Spain	Non-native	Present work
OQ193175	Passeig Maritim de la Barceloneta, Barcelona	Spain	Non-native	Present work
MW520550	Porto Santo	Portugal	Non-native	Stüben et al. (2021)
HM433615	Not available	Virgin Islands	Non-native	Direct submission to GenBank
KU896925	Not available	Virgin Islands	Non-native	Chamorro et al. (2016)
KU896926	Not available	Virgin Islands	Non-native	Chamorro et al. (2016)
KU896928	Not available	Virgin Islands	Non-native	Chamorro et al. (2016)
KU896931	Not available	Virgin Islands	Non-native	Chamorro et al. (2016)
KU896932	Not available	Virgin Islands	Non-native	Chamorro et al. (2016)

computed both for the native and the invaded ranges of *S. acupunctatus* through Mega XI (Tamura et al. 2021). A Templeton, Crandall and Sing (TCS) parsimony Network (Clement et al. 2000) connecting haplotypes was obtained with popART (<http://popart.otago.ac.nz>, Accessed on 20.12.2022) with the aim to visualise the relationship amongst the new and previously-described mitochondrial haplotypes (see Sciandra et al. (2022)).

Results

Species distribution

Overall, the agave weevil was reported on all continents, except for Antarctica. Based on genetic analyses and literature, the native range of this species includes the USA, Mexico and, most likely, the rest of continental Central America (Vaurie 1971). The invasive range of this species (Fig. 1a) includes four South American countries (Brazil, Colombia, Ecuador and Venezuela), the insular USA (including Hawaii and Virgin Islands), Caribbean islands, southern European countries (Portugal including Madeira, Spain including Canary and Balearic Islands, Italy including Sardinia, Sicily and several small islands, Greece including Aegean Islands, Croatian islands and Cyprus), South Africa, Kenya, Tanzania, Saudi Arabia, Java, Sumatra, Borneo and southern Australia (Fig. 1b). Occurrences from other countries (i.e. Israel, the Netherlands, UK and Argentina) were not confirmed and may represent single interceptions.

Species distribution models

Projections of each statistical model (Suppl. material 1: fig. S5) produced slightly different results that were averaged in the ensemble model. The ensemble model for current climate showed a high climatic suitability in the native range and in some parts of the non-native range, i.e. the eastern areas of South Africa, the northern Rift Valley (i.e. from Eritrea and Ethiopia to Tanzania), parts of South America and the central and western Mediterranean countries (Fig. 2a). Highly suitable areas were also predicted in southern Australia, where *S. acupunctatus* has been scarcely recorded so far, the area around the Caspian Sea and the Middle East, where the weevil has not yet been recorded (Fig. 2a).

Considering future climate scenarios forecast for 2070, the areas suitable for *S. acupunctatus* would increase especially towards temperate-cold latitudes, both in Europe and worldwide (Fig. 2b).

Values representing the degree of climatic similarity between future and present conditions are shown in Fig. 2c, with an increase in suitability of 72.62% and a decrease of 27.43%, based on the number of cells around the Globe. There was agreement between the different algorithms used to predict the species' distribution under future climates (Fig. 2d; Suppl. material 1: fig. S5). The climate suitability of most temperate areas of both Hemispheres will increase for *S. acupunctatus* in the future.

The MESS analysis showed that the projection area shared a medium to high environmental similarity with many countries in the training area, except for a few northern Eurasian areas (Suppl. material 1: fig. S6).

Genetic analyses

The COXI sequences were obtained from all analysed samples. All sequences generated in the present study were deposited in GenBank (Table 2). The alignment of COXI gene

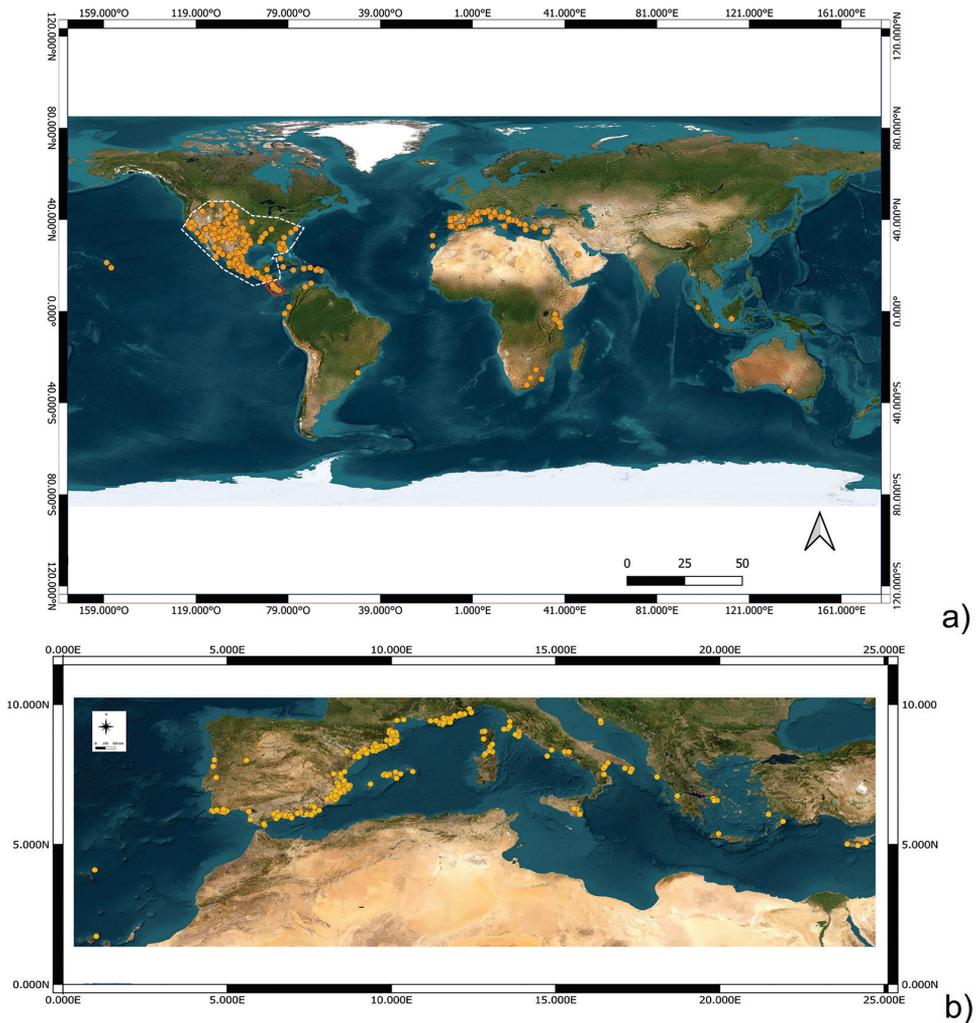


Figure 1. **a** Worldwide distribution of *Scyphophorus acupunctatus* in both native (central and southern North America) and non-native ranges ($n = 1135$ occurrences) **b** distribution of *S. acupunctatus* in southern European Countries (orange dots refer to occurrence sites of agave weevil). The white dotted line includes occurrences from the native range, whereas the solid red line includes occurrences of uncertain origin. Occurrences outside dotted lines are non-native populations. Sources: Data SIO, NOAA, US Navy, NGA, GEBCO 2016 TerraMetrics 2016 Google; Wikimedia Commons, user Norman Einstein, CC-BY-SA-3.0.

consists of 627–903 nucleotides for 65 individuals, including 32 from the Mediterranean area. All individuals belonged to *S. acupunctatus*, as no record of *S. yuccae* was confirmed in the analysed samples nor in any sequence deposited in the GenBank. Nucleotide and haplotype diversity was lower in the alien than in the non-native range (Table 3).

An ML tree is presented in Fig. 3 and supports the monophyly of *S. acupunctatus* (Fig. 3). Samples from the native range (Mexico and Continental USA) clustered to-

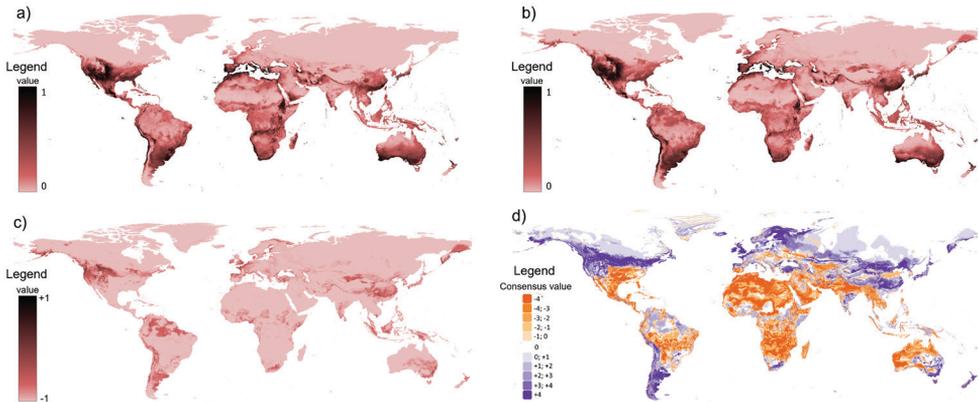


Figure 2. **a** Current potential distribution of *Scyphophorus acupunctatus* worldwide (suitability increasing from pink to black) **b** future potential distribution of *S. acupunctatus* under climate projections using the global climate model for 2070 (suitability increasing from pink to black) **c** differences between future and present conditions [future-current] for the RCP 2.6 scenario obtained by subtracting, for each cell, the predicted suitability under current climate from that under future climates. Pink to black: increase in climatic suitability in the future **d** consensus change for RCP 2.6 scenario. Dark blue (+4) indicates that all models predicted an increase in suitability, whereas dark orange (-4) indicates a full agreement in predicting a decrease in suitability; white indicates disagreement across models (0 value).

Table 3. Indices of genetic diversity for native and most-likely native ($n = 26$ samples) and non-native ($n = 39$ samples) populations of *Scyphophorus acupunctatus* (cf. Table 2).

	Total	Native and most-likely native populations	Alien populations
π (nucleotide diversity index \pm standard deviation)	0.22 ± 0.05	0.59 ± 0.05	0.03 ± 0.01
h (haplotype diversity index \pm standard deviation)	0.42 ± 0.15	0.61 ± 0.19	0.09 ± 0.01
Number of segregating sites	170	161	115
Number of Parsimony Informative sites	154	148	71

gether and represented the sister group of the clade that included samples from southern countries of Central America (Costa Rica and Guatemala) and all the non-native range (Fig. 3).

The TCS network highlighted that the majority of introduced individuals in Sardinia, Sicily, Corsica, continental Italy (Latium and Liguria), continental France, Spain and Portugal belonged to the same haplotype, as in Costa Rica and Guatemala (Fig. 4).

Discussion

This study showed for the first time the actual and potential global distribution of the agave weevil, both in the native and non-native ranges and assessed the phylogenetic relationships between native and non-native populations at the global scale.

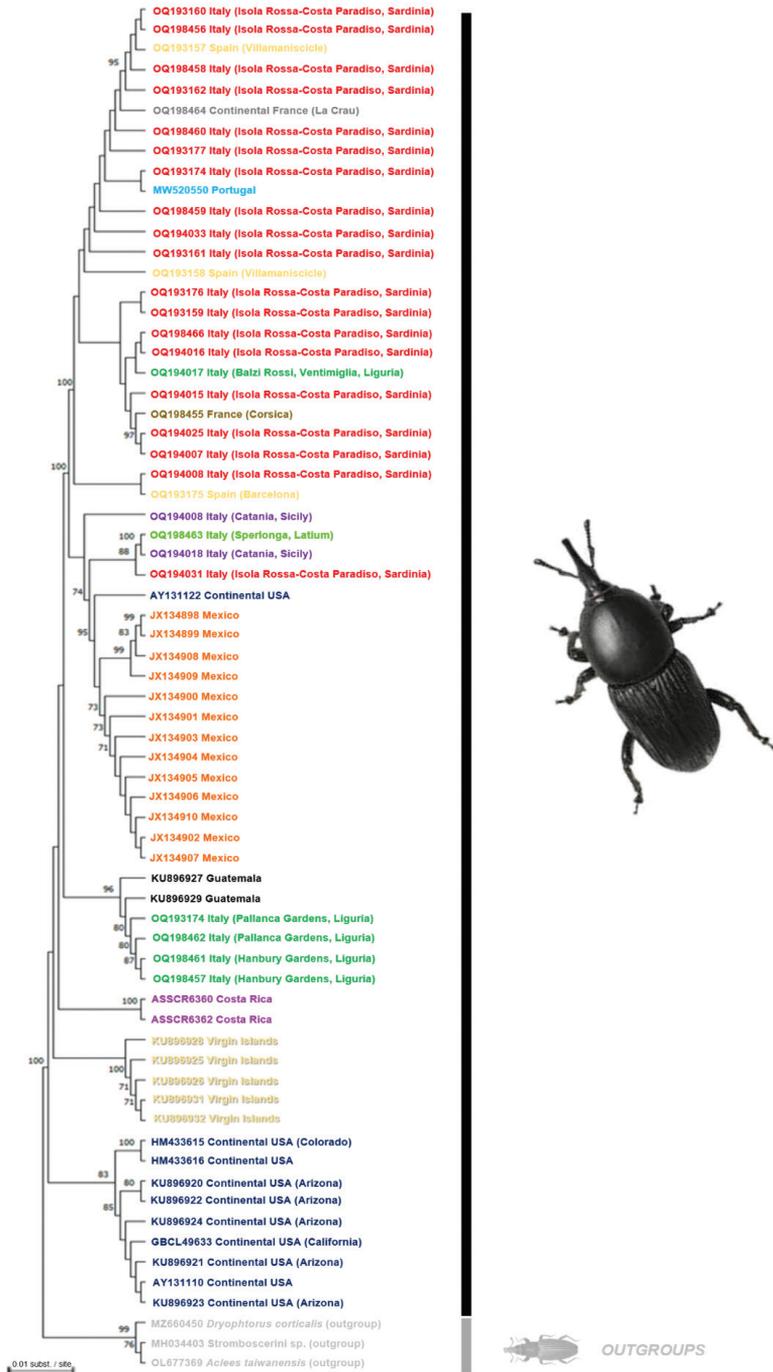


Figure 3. Maximum Likelihood (ML) phylogenetic tree obtained from the analysis of COXI for 65 individuals of *Scyphophorus acupunctatus* ($n = 39$ from non-native range, $n = 22$ from native range, $n = 4$ from most-likely native range, cf. Table 2). The statistical support of major clades is shown at their nodes (NJ Bootstrap support/Bayesian probabilities/ML Bootstrap support).

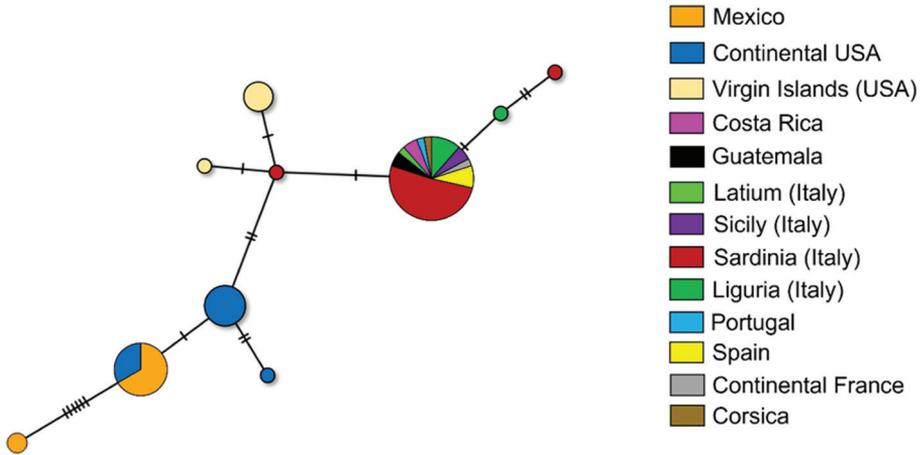


Figure 4. *Scyphophorus acupunctatus* TCS Network showing the relationship amongst mitochondrial COXI haplotypes. Circles represent different haplotypes ($n = 10$). Circle size is proportional to the number of samples for each haplotype. Mutations are shown as hatch marks.

The presence of this species was confirmed in several countries, whereas some of those listed in CABI's overview of invasive species (the Netherlands, UK, Israel, New Zealand and Argentina: CABI (2023)) were not confirmed. In contrast, the occurrence of non-native *S. acupunctatus* was reported for the first time in Ecuador, through the iNaturalist repository, as well as in many Italian regions where this weevil was previously not reported (Calabria, Sardinia, Piedmont and Campania: Suppl. material 1: fig. S7). In particular, the first record of this weevil in Sardinia, in the north-western part of the island, was due to detailed and addressed research by the authors of this work.

Despite being reported as the most important pest for agave species (Waring and Smith 1986), the agave weevil is not commonly identified by the public; thus, it is unsuitable for citizen-science surveys (cf. Mazza et al. (2020) for *C. ayyari*). Accordingly, most data on the distribution of this species were obtained from scientific research and insect monitoring projects conducted by specialists (Kontodimas and Kallinikou 2010; Vassiliou and Kitsis 2015). The occurrence of the agave weevil was also confirmed in all the other regions where it was previously reported, i.e. Sicily, Basilicata, Apulia, Latium, Tuscany and Liguria, as well as some small Thyrrhenian islands (i.e. Giglio, Elba, Giannutri and Ponza).

The presence of the agave weevil in other Italian peninsular regions along the coastline (e.g. Molise, Abruzzo, Marche, Emilia Romagna and Veneto) cannot be ruled out. Thus, a focused monitoring programme is required, particularly in late spring and during the daytime, when most observations occur (López-Martínez et al. 2011; Figueroa Castro et al. 2013).

Species distribution modelling showed a high climatic suitability for this species throughout the Mediterranean Basin, potentially increasing with increasing temperature and decreasing precipitation, i.e. with the ongoing climatic change. Accordingly, the native range of *S. acupunctatus* currently includes mostly dry areas of Central

America, also suggesting the adaptation of this insect to hot desert areas (including mountainous ones), where most Agavaceae, i.e. succulent plants representing the staple of its diet and reproductive sites, grow. The distribution of *S. acupunctatus* in Europe and Africa is linked to the distribution of Agavaceae and Dracaenaceae as ornamental plants. Particularly, in the Mediterranean countries, these plants mostly occur in botanical gardens and along the coastline, i.e. where most records of *S. acupunctatus* have been reported (Smith and Figueiredo 2007; Celesti Grapow et al. 2016; Cascone et al. 2021).

Genetic analyses showed a strong genetic uniformity for the non-native populations. A lower nucleotide and haplotype diversity was observed in the non-native range compared to the native range, possibly due to a founder effect. The presence of a single widespread haplotype in Europe suggested that most of the introductions may have originated from a small geographical area in Central America or a small number of introduction events occurred. This contrasts with other species, which were introduced through multiple unintentional introductions in Europe. These include *C. ayyari*, *H. halys* and *Megachile* (*Callomegachile*) *sculpturalis* Smith, which show a high genetic diversity linked to several introduction events (Cesari et al. 2018; Mazza et al. 2020; Lanner et al. 2021). *Scyphophorus acupunctatus* in Europe may have originated from one or a few introduction events from Central America (most likely from Guatemala or Costa Rica) through the ornamental plant trade (Global Invasive Species Database 2023). This is in line with EPPO's report (EPPO 2022b), which traces the source of the first introduction of agave weevil to Italy to countries of southern Central America, based on interception data.

Drawing definite conclusions from single-gene analyses may be misleading. However, the largest genetic library for *S. acupunctatus* built in the present study may serve as a comparison for future studies and for species identification (Azuara-Domínguez et al. 2013; Chamorro et al. 2016). All analysed samples belonged to *S. acupunctatus* and the only deposited sequence of the sister species *S. yuccae* on GenBank suggests that this species could be a synonym to *S. acupunctatus*, as already hypothesised by Chamorro et al. (2016).

In general, our data showed a high climatic suitability for *S. acupunctatus* in Eurasia and Africa (particularly in the Mediterranean Basin coastline), including areas where this weevil is not yet present. This suggests that if no management actions are taken to limit its spread, there is potential for range expansion towards continental and temperate Europe in the upcoming years. Given the impacts on cultivated agave plants, early detection of this species in new areas should be promoted to prevent further invasions, by means of free online citizen-science platforms and coordination of phytosanitary services and national institutions for the prevention of biological invasions.

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Supplementary material I

Supplementary data

Authors: Andrea Viviano, Arturo Cocco, Paolo Colangelo, Giuseppe Marco Delitala, Roberto Antonio Pantaleoni1, Laura Loru

Data type: docx

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Assessing the invasion potential of five common exotic vine species in temperate Australian rainforests

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Abstract

To compare the capacity of native and exotic vine species established under a rainforest canopy, a comparison of growth rates and resource allocation was made amongst five exotic vine species that are serious and common invaders and two common native vine species under two light conditions reflective of edge and interior canopy conditions. All species experienced heavy reductions in growth parameters in the low-light treatment, but three exotic species showed stronger growth under the low light. All exotic species had higher plasticity in leaf morphology showing a significant increase in SLA under low light. Native vines may have a lower capacity to change leaf morphology in shade, as a result of local adaptation to edge habitats. Higher SLA under both low and high light conditions suggests that exotic vine species are able to exploit a range of forest conditions better than the native species. Three species, *Anredera cordifolia*, *Araujia sericifera* and *Cardiospermum grandiflorum*, appear particularly capable of invading rainforest interiors. Individuals produced few leaves, focusing resources on roots and stems suggesting a response to reach the canopy quickly. With their long-distance seed dispersal, plasticity in leaf SLA and high RGR, these species appear most likely to invade undisturbed rainforest.

Keywords

Anredera cordifolia, *Araujia sericifera*, *Cardiospermum grandiflorum*, *Cissus antarctica*, *Delairea odorata*, *Ipomoea cairica*, low light, *Pandorea pandorana*, SLA

Introduction

In forests, vines can cause structural damage to the canopy, reduce light availability and increase competition for underground resources, which results in reduced growth and survival of host and neighbouring trees (Estrada-Villegas and Schnitzer 2018). This fast growth strategy is achievable as vines do not invest biomass into self-supporting structures and therefore, they invest more in growth and reproductive structures. Compared to other woody plants, vines tend to be situated on the “faster end” of the life-history spectrum (Westoby et al. 2004), through having higher specific leaf area (SLA) (Llorens and Leishman 2008; Mello et al. 2020), higher photosynthetic capacity (Mello et al. 2020) and more investment into stem growth (Ichihashi and Tateno 2015). The difference in traits between vines and trees, as well as the negative impacts of vines on trees is well studied, especially in tropical systems (Ingwell et al. 2010; Estrada-Villegas and Schnitzer 2018; Mello et al. 2020); however, there is also overlap in trait values between some vines and trees, as well as amongst species of invasive and native vine species (Llorens and Leishman 2008; Osunkoya et al. 2010, 2014; French et al. 2017; Mello et al. 2020).

In a meta-analysis of 117 studies, Van Kleunen et al. (2010) compared traits across studies comparing invasive and native plants species that co-occurred, many co-familial. Invasive plant species had higher SLA, higher photosynthetic capability and faster growth rates than native species (Van Kleunen et al. 2010). There is some evidence that the pattern is also true when vines are considered separately. Greenhouse studies in tropical areas (Osunkoya et al. 2010, 2014) showed that four common invasive vine species in eastern Australia have higher photosynthetic capability, SLA and phenotypic plasticity than for common functionally similar native species, although relative growth rates were not different. This suggests that invasiveness is associated with a set of traits that provide a competitive advantage. However, there are other factors that influence invasiveness, such as release from herbivores (Keane and Crawley 2002) and whether the invasive environment is adequate to successfully reproduce and disperse (Van Kleunen et al. 2015). We predict that, if native vines show similar growth and plasticity to invading exotic invasive vines, then other factors, such as release from herbivores and pathogens may be particularly important to allow them to invade and become abundant. Furthermore, if a particular set of traits are critical in invasiveness, then we would predict that native species which can dominate sites and be extremely abundant would show similar invasive traits. A study in a temperate, moist sclerophyll forest found little difference in above-ground biomass allocation between invasive and native vine species (Llorens and Leishman 2008), which could indicate that other factors give exotic invasive vines a competitive advantage. Furthermore, there was no difference found in the height reached or percentage of host height reached between exotic invasive species and native species, suggesting that there was little difference in impact on the community between exotic invasive and native vine species.

Increased investment in growth can be allocated to roots, stems or leaves. Higher investment into stem elongation can be particularly beneficial as it allows the vine

to reach higher into the canopy quickly and, thus, gain more light for photosynthesis (Falster and Westoby 2003; Llorens and Leishman 2008). However, investment into stem elongation must be balanced adequately with the other needs of the vine to survive and reproduce. If exotic invasive vines are released from herbivores, then stem elongation to get their leaves into the light would be the most beneficial strategy. Therefore, we predict that exotic invasive vine species are likely to invest more into stem growth than leaf growth compared to native vine species.

While vine invasion is particularly problematic at the light-filled edges of rainforest patches, the ability to grow and establish in the forest interior would be an invasive characteristic that increases the risk and impacts of that species in closed forests. The plasticity to change growth parameters in low-light situations is, thus, an important part of identifying exotic invasive species that pose the greatest risk. Some invasive vine species are considered more problematic in forests than others due to apparent high growth rates or high propagule pressure. In Australia, there are at least 179 species of exotic vines (Harris et al. 2007), though not all are considered equal threats to biodiversity. It is likely that the species that should be considered to be greater threats should have higher photosynthetic ability, invest more into stem elongation and, consequently, have higher growth rates as well as show high rates of growth even in interior light conditions. Furthermore, knowledge of how invasive and native vine species differ in these traits with light availability can help to predict the species that can exploit disturbances better or can invade undisturbed interiors. At present, information on growth rates and resource allocation under differing light levels is poorly understood for all exotic vine species in Australia, preventing good risk assessments and prioritisation in management. Collectively, invasion and establishment of exotic vines and scramblers are listed as a key threatening process in New South Wales (NSW) where 38 entities are listed as affected by vines and scramblers. However the list of vines and scramblers includes species that are much less abundant and problematic and some that occur frequently (A. Bernich and K. French, unpublished data).

In order to help evaluate the invasiveness of key exotic invasive vine species, we assessed growth rates of five common invasive exotic vine species in eastern Australia and two common native species on host trees in a shade house with two shade treatments. We measured relative growth rates (RGR), stem lengths, proportion of biomass allocated to leaves, stems and roots and specific leaf area. We predicted that individuals of each species grown in less shade will have higher RGR and longer stem lengths, though lower SLA. We predicted that invasive species will also have higher RGR and stem lengths and higher SLA indicating higher photosynthetic efficiency, which then leads to relatively less investment in leaves and more relative investment into stems and roots compared to native species. We also predicted that differences in trait values would occur amongst species and show that some species are able to exploit establishment opportunities under the rainforest canopy. One of the native species, *Cissus antarctica*, is especially abundant at the edge of disturbed rainforests in eastern Australia where it can dominate and smother canopy causing significant harm to native host species. There is concern that such dominance may cause forest interiors to become degraded.

Methods

Study species

We grew seven vine species, five exotic invasive species (*Anredera cordifolia*, *Araujia sericifera*, *Cardiospermum grandiflorum*, *Delairea odorata* and *Ipomoea cairica*) and two native species (*Cissus antarctica* and *Pandorea pandorana*). All species are commonly found in rainforests, wet sclerophyll forests and disturbed sites on the east coast of Australia. *Anredera cordifolia* (family Basellaceae) is a semi-succulent twiner from South America, that is listed as a Weed of National Significance in Australia. It was introduced in the early 1900s (Vivian-Smith et al. 2007) and quickly establishes at sites through vegetative reproduction via aerial tubers. *Araujia sericifera* (family Apocynaceae) is a South American twiner also introduced in the early 1900s, which disperses by producing masses of windblown seeds. *Cardiospermum grandiflorum* (family Sapindaceae) is a tendril climber from South America, introduced in the 1920s, with multiple seeds in a papery “balloon” that can be distributed by wind and water. *Delairea odorata* (family Asteraceae) is an herbaceous twiner or scrambler from Africa that was introduced in the early 1900s, that spreads locally through vegetative reproduction (stolons and stem fragments), although it also produces viable seeds in Australia. *Ipomoea cairica* (family Convolvulaceae) is a twiner from tropical Africa and Asia and was introduced in the 1840s, it is common in coastal eastern Australia and can withstand a wide range of environmental conditions (Liu et al. 2016a). The native *Cissus antarctica* (family Vitaceae) is a tendril climber that can grow thick stems (up to 15 cm in diameter) and is abundant along the east coast of Australia, producing a fleshy fruit. *Pandorea pandorana* (family Bignoniaceae) is a large woody twiner that is also abundant in eastern Australia, producing winged seeds.

Vine species were all collected from forests near Wollongong, with some species being grown as ~ 30 cm cuttings (*D. odorata*, *I. cairica*, *P. pandorana* and *C. antarctica*), from seeds (*C. grandiflorum*), tubers (*A. cordifolia*) or harvested seedlings (*A. sericifera*) which had the first two true leaves, around 5–15 cm in height. We attempted to grow both *C. grandiflorum* and *A. sericifera* as cuttings, though no *C. grandiflorum* cuttings were successful and there was only a 10% success rate for *A. sericifera*. Propagules for all species were collected in September 2021 and were grown until sufficient individuals were established to be used in the experiment. All propagules were collected along forest edges (i.e. tracks or clearings) which were more representative of the medium light treatment (see below). As establishment times varied amongst species, the date that species were potted and placed next to a host tree occurred over two months at the beginning of the Austral Summer (late October – mid December).

All vine individuals were grown on *Acmena smithii* (cultivar ‘Speedy Screener’, family Myrtaceae) host trees that were potted in 300 mm pots. The host trees ranged in height from 1 m to 1.8 m tall. *Acmena smithii* is a common tree in eastern Australia that grows in rainforests and wet sclerophyll forests.

Experimental design

All vines and host trees were grown in a shade house at the University of Wollongong, NSW 34.4054°S, 150.8784°E. The shade house had two sections, one with low light penetration to mimic the understorey under a rainforest canopy and one with medium light to mimic a gap in a rainforest or rainforest edge. The roof of the low light section was covered in two layers of shade cloth, which allowed 2% of light to reach the floor (similar to 85–95% canopy cover), whereas the medium light section had one layer of medium shade cloth, which allowed 30% of light to reach the floor (similar to 50–60% canopy cover).

Six individuals of each species were randomly selected for the medium light and low light treatments and were transplanted into 300 mm pots filled with commercial potting mix (Osmocote Premium) and given 25 g of slow-release fertiliser (Power-Feed 500 g All Purpose Controlled Release). They were then placed adjacent (on the southern side) to an *A. smithii* individual in their allocated shaded areas. Two to four extra individuals were harvested and dried in an oven at 65 °C for five days to measure dry biomass of roots, stems and leaves at the start of the experiment (the difference in number of individuals for each species was due to the death of some individuals before they could be dried out). Vines and trees were watered by an automatic dripper system attached to a tap timer, with each plant having a dripper spike in the soil of the pot. Plants were drip-watered for 10 minutes at 6 am and 6 pm every day.

The experiment for each species began when plants were placed next to the host plant. Initial plant sizes are shown in Suppl. material 3. Vines were grown for 24 weeks, after which time, vines were removed from host trees and laid horizontally on the ground to measure the longest stem (from the junction of the roots and the stem to the tip of the longest stem, to give a measurement of the potential height each individual could reach). Ten leaves for each vine individual were removed and measured using a Li-Cor leaf area meter (Model Li-3000A, Lincoln, Nebraska, USA), then put into separate labelled bags for drying (65 °C for 5 days), before being weighed and specific leaf area calculated (SLA = leaf area/dry weight). Then, each individual was sorted into leaves, stems, aerial tubers (for *A. cordifolia*) and roots, with roots being washed to remove soil, before being placed in a drying oven at 65 °C for 5 days and then weighed. Relative growth rate (RGR) was calculated using the formula:

$$\text{RGR} = \frac{\ln DW_f - \ln DW_i}{\text{no. of days}}$$

where DW_f is the total dry weight at the end of the experiment for an individual and DW_i is the average dry weights of the plants sacrificed at the beginning of the experiment for the species being tested.

The dry weights of each plant part (roots, stems and leaves) were divided by the total dry weight to give percentages of biomass allocation; these parameters are referred

to as root mass fraction (RMF), stem mass fraction (SMF) and leaf mass fraction (LMF). For *A. cordifolia*, aerial tuber weight was added to RMF as a measure of investment into energy storage; however, the proportion of biomass invested into aerial tubers by *A. cordifolia* was also recorded separately. Traits were only measured on individuals that did not die in the experimental period.

Data analysis

We used a Bayesian modelling approach to estimate the distribution of values for each of the measured plant variables for each combination of species and shade treatment. The fitted distributions were then used to estimate the magnitude and direction of differences in response between species within each treatment and between treatments for each species. Stem height and SLA values, which could only be positive, were modelled as gamma-distributed variables with the shape parameter of the distribution being allowed to vary between shade treatments. RGR values were modelled as being drawn from a Student-t distribution since values could be negative and some outliers were evident in the observed data. The shape (degrees of freedom) parameter of the distribution was treated as an unknown quantity to be estimated by the model, while the scale parameter (standard deviation) was allowed to vary between treatments. The proportion of biomass allocated to each of leaf, stem and root fractions was modelled using Dirichlet regression.

Models were fitted by Hamiltonian Monte Carlo sampling via the “brms” package version 2.18 (Bürkner 2017) in R version 4.2.1 (R Core Team 2022). For all models, we set weakly informative prior distributions for parameters, as recommended by Gelman (2009) and van de Schoot (2021), to ensure that fitted distributions reflected the observed data while constraining the model fitting process from exploring unrealistic ranges of parameter values. For the Dirichlet regression of biomass allocation fractions, we explored alternative choices for prior distributions on the intercept and regression coefficients using prior predictive simulation. This involved fitting the model, based only on candidate prior distributions, i.e. with no observed data. The simulations highlighted the potential sensitivity of model predictions to the choice of standard deviation for the Normal priors on the intercept and regression coefficients. Given this, we chose to treat the prior standard deviation as an additional parameter to be learned from the data and set an exponential hyper-prior distribution on it.

For each model, we ran four Markov chains with 5000 iterations and 1000 warm-up iterations. Model convergence was assessed using the Gelman-Rubin statistic, which showed convergence for all models and by checking for an adequate number of effectively independent samples to ensure reliable estimates of the tails of the fitted distributions. In addition, we graphed posterior model predictions together with observed data values for each measured variable to check for any disagreement that might indicate a problem with model structure or convergence.

For all models other than stem growth rate, the distribution of differences in response between each pair of species within each shade treatment was estimated by subtracting posterior predictions of mean response for one species from those for the

other species. For the stem growth rate model, difference calculations were based on posterior predictions of median rather than mean response as some observed values were close to zero, which resulted in a strongly right-tailed posterior distribution for which the median is a more representative summary statistic.

Results

At the end of the six months, two *A. sericifera* individuals had died in the medium light treatment. In the low light treatment 10 deaths occurred: three *I. cairica* individuals, two *D. odorata*, three *A. sericifera*, one *C. antarctica* and one *P. pandorana*.

Relative growth rate

For all species, mean RGR was consistently higher when grown under medium light compared to low light, with no overlap in the 95% range of predicted mean RGR values (Fig. 1, Table 1). Two exotic vines did poorly in low light, *D. odorata* (93% reduction in mean predicted RGR) and *I. cairica* (75% reduction) and the two native spe-

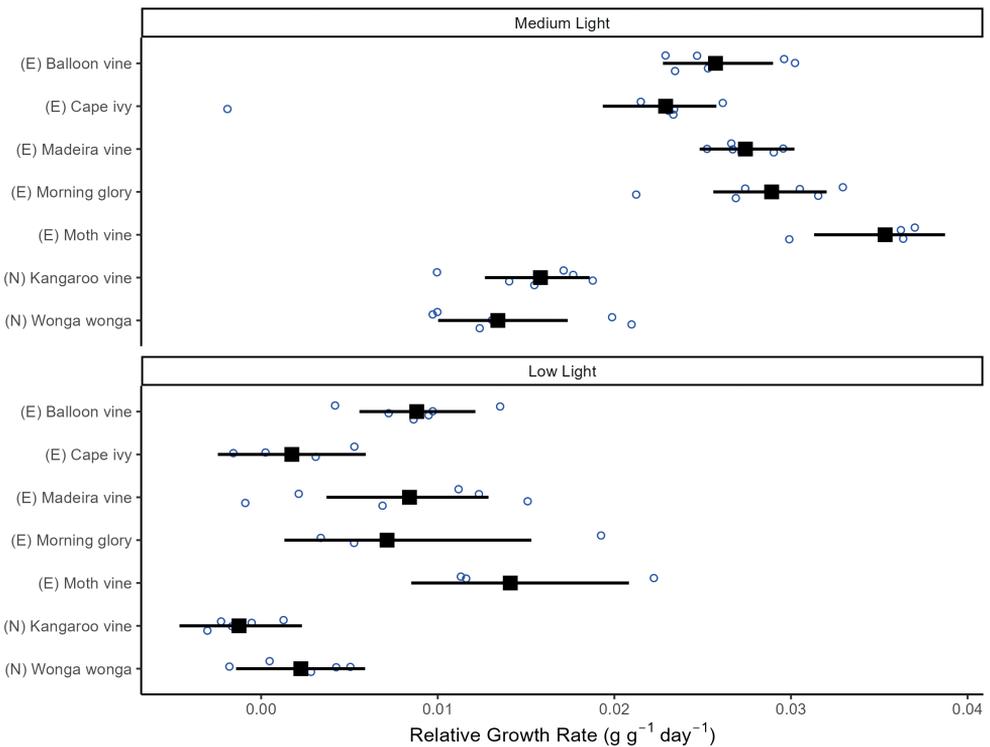


Figure 1. Mean relative growth rate (RGR) with 95% bounds on the mean predicted values from each model (black lines) and observed RGR value for individual plants (blue dots) for both light treatments. (E) are exotic vines, (N) are native vines.

Table 1. The predicted mean trait values and the 95% bounds on the mean predicted values from each model (smaller font). RGR = relative growth rate ($\text{g g}^{-1} \text{day}^{-1}$), stem growth = increase in stem length per day (cm/day), LMF = leaf mass fraction (%), RMF = root mass fraction (%), SMF = stem mass fraction (%), SLA = specific leaf area (cm^2/g), (E) denotes exotic species, (N) denotes native species. * note that RMF for *Anredera cordifolia* includes the weight of aerial tubers.

Species	Light treatment	Predicted mean trait values					
		RGR	Stem growth	LMF	RMF	SMF	SLA
<i>Anredera cordifolia</i> (E)	Medium	0.0274	1.55	16.5%	65.8%	17.7%	323.0
		0.0248–0.0302	1.29–2.31	14.9–22.5	44.3–56	26.3–36.2	272.7–379.4
	Low	0.0084	0.47	32.9%	42.5%	24.7%	988.1
<i>Araujia sericifera</i> (E)	Medium	0.0353	1.14	18.8%	37.0%	44.3%	255.0
		0.0313–0.0387	0.82–2.05	13.3–23.7	30.8–44.1	37.6–51.4	209.83–302.27
	Low	0.0141	0.24	11.3%	52.7%	36.0%	625.8
<i>Cardiospermum grandiflorum</i> (E)	Medium	0.0257	1.62	24.4%	24.1%	51.5%	329.5
		0.0227–0.0290	1.25–2.71	19.7–29.3	19.4–29.1	45.7–57.2	281.2–383.9
	Low	0.0088	0.48	42.2%	12.6%	45.2%	745.7
<i>Delairea odorata</i> (E)	Medium	0.0229	1.92	15.3%	12.5%	72.3%	570.4
		0.0193–0.0258	1.46–3.20	11.6–19.4	9.2–16.6	66.7–76.9	480.4–663.3
	Low	0.0017	0.62	25.6%	12.1%	62.3%	1607.5
<i>Ipomoea cairica</i> (E)	Medium	0.0289	2.62	11.9%	45.7%	42.5%	379.3
		0.0256–0.0320	2.02–4.25	9.1–16.1	39.6–51.0	36.5–48.0	321.5–443.6
	Low	0.0071	0.74	29.6%	33.5%	36.9%	1219.6
<i>Cissus antarctica</i> (N)	Medium	0.0158	0.80	45.6%	13.4%	41.0%	189.8
		0.0127–0.0186	0.61–1.32	38.5–50.3	10.5–18.2	36.0–47.1	161.6–219.9
	Low	-0.0013	0.11	45.2%	25.3%	29.6%	362.7
<i>Pandorea pandorana</i> (N)	Medium	0.0134	0.91	30.8%	24.6%	44.6%	281.7
		0.0100–0.0174	0.69–1.54	25.2–35.6	20.2–30.0	38.9–50.5	242.1–327.8
	Low	0.0022	0.20	42.3%	28.5%	29.2%	482.6
	-0.0014–0.0059	0.14–0.58	31.1–53.9	18.5–39.6	19.0–39.9	389.1–585.4	

cies, *P. pandorana* (84% reduction) and *C. antarctica* (~ 98% reduction) showed large reductions in RGR under low light. *C. antarctica* barely grew in the low light treatment making estimates of percentage reductions difficult to calculate. The three other exotic species had reductions of between 60 and 69%. Higher variability in RGR was seen amongst individuals of exotic invasive species in the low light (see Suppl. material 1).

All the exotic invasive species grown in the medium light treatment had higher predicted mean RGR values than the two native species (Fig. 1, Table 1). *A. sericifera* had the highest predicted mean RGR with only *I. cairica* having a slight overlap in the 95% range of predicted mean values. All other exotic invasive vine species had relatively similar predicted mean RGR values with a high percentage of overlap in the 95% bounds on the predicted mean. In the low light treatment, *A. sericifera* still tended to have the highest RGR, though the 95% bounds on the predicted mean substantially overlapped with three of the other exotic invasive species (Fig. 1, Table 1, Suppl. material 2).

Length of main stem

Similar to RGR, the predicted median stem length grown per day for all species in the medium light treatment was greater than the low light treatment, with no overlap in the 95% bounds on the predicted median values from the model (Fig. 2). In the medium light treatment, *I. cairica* had the highest predicted median stem growth per day at 3.2 cm/day and had final raw stem lengths across individuals of 3.6–8.5 m. *D. odorata* also had high stem growth rates, with a slight overlap on the 95% bounds on the predicted median with *I. cairica* and final stem lengths of 3.9–6.4 m. *A. sericifera* had the lowest predicted median stem growths for the exotic invasive species. The two native species clearly had the lowest predicted median stem growth (Fig. 2), with there being a slight overlap in the 95% bounds on the predicted median with *A. sericifera* only (Suppl. material 1).

In the low light treatment, *I. cairica* and *D. odorata* had the highest predicted median stem growth per day with high variability amongst individuals. Other species had closer predicted median values. The gap between exotic invasive and native species in the low light treatment was actually higher than the medium light treatment (Fig. 2), though *A. sericifera* only had slightly higher predicted stem growth than *P. pandorana* at 4 mm per day (Fig. 2). Mean predicted values were very similar to median values (Fig. 2, Table 1).

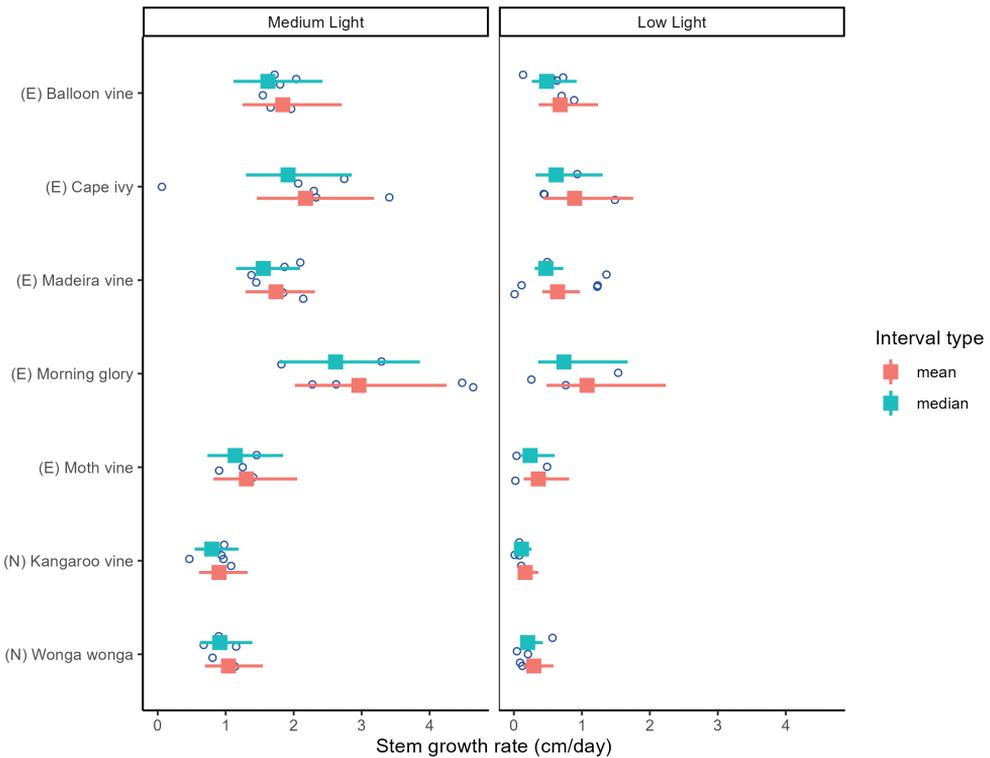


Figure 2. Median (blue lines) and mean (red line) stem growth rate with 95% bounds on the mean predicted values from each model, for both light treatments. Blue dots show the observed value for individual plants. (E) are exotic vines, (N) are native vines.

Biomass allocation

The percentage of biomass invested into leaves, stems and roots differed amongst species and light treatments (Fig. 3, Table 1). In the medium light treatment, exotic invasive species invested very little into leaves, with all species having a predicted mean proportion of leaf biomass less than 25%, while the two natives, *C. antarctica* and *P. pandorana* had predicted mean leaf biomass proportions of 47% and 31%, respectively. *Anredera cordifolia* and *I. cairica* invested relatively highly into root biomass with the predicted mean being 66% and 46%, respectively. For *A. cordifolia* in the medium light, the mean number of aerial tubers produced was 45.3 (range 29–61). Aerial tubers accounted for between 5 and 30% of the total biomass (mean = 15%). The roots, excluding aerial tubers, accounted for, on average, 55% of total biomass. No aerial tubers were produced by individuals grown in the low light treatment. Two species, *D. odorata* and *C. antarctica* invested very little into roots (Fig. 3). *Delairea odorata* invested the greatest amount into stems (predicted mean of 72%) (see Suppl. material 1).

Biomass allocation for all species in the low light treatment was more varied, seen by wider 95% bounds on the predicted mean values (Fig. 3). Natives did invest more biomass in leaves compared to moderate light treatments, but overall, the allocation to

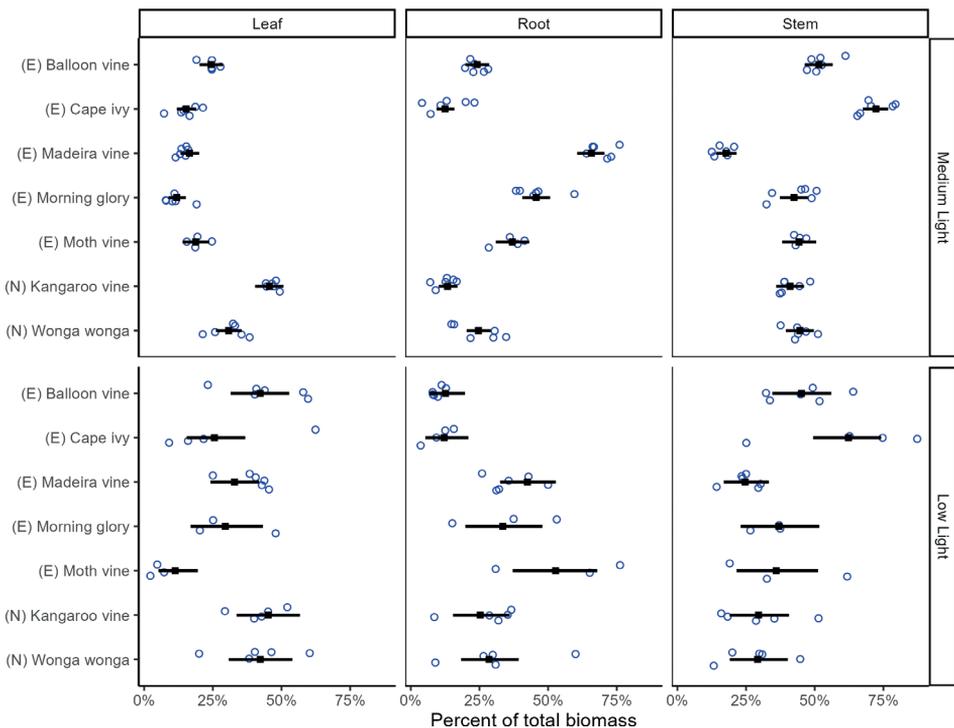


Figure 3. Mean proportion with 95% bounds (black lines) on the mean predicted values from each model, of biomass invested into leaves, roots and stems for all species in both light treatments. Blue dots are observed proportions for individual plants.

leaves across all vines was similar. *A. sericifera* tended to allocate resources into stems and roots rather than leaves. *Delairea odorata* and *C. grandiflorum* invested very little in roots, focusing on stem growth by comparison (Fig. 3, Table 1, Suppl. material 2).

Specific leaf area

The 95% bounds on the mean predicted range for SLA was substantially higher in the low light treatment than the medium light for all species (Fig. 4, Table 1), with there being no overlap in the 95% bounds on predicted means between treatments for each species (Suppl. material 2). The greatest difference in predicted mean SLA between the treatments was seen in *I. cairica* (222% difference) and *A. cordifolia* (206%). *D. odorata* (182%), *A. sericifera* (145%) and *C. grandiflorum* (126%) had a moderate predicted increase in SLA, while the two natives had the lowest difference in SLA amongst treatments; *C. antarctica* (91%), then *P. pandorana* (71%).

In general, exotic invasive species had higher SLA than native species, except for *A. sericifera* in the medium light which had a considerable overlap in the 95% bounds on predicted mean values with *P. pandorana*, though only a slight overlap in the low

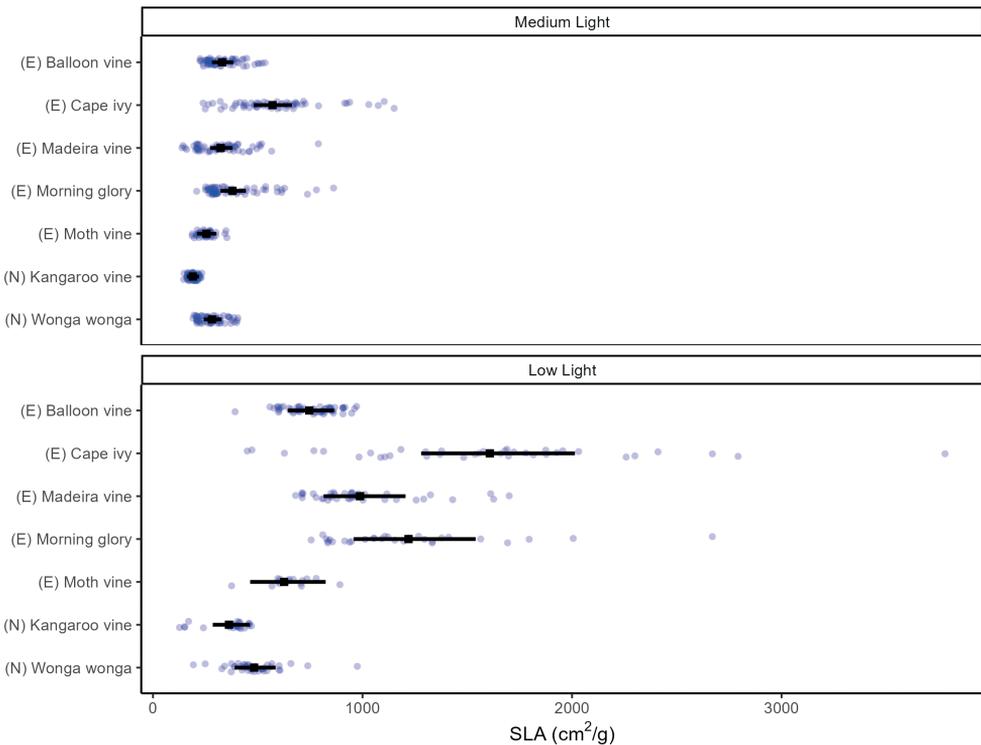


Figure 4. Observed specific leaf area (SLA) with 95% bounds (black line) on the mean predicted values from each model for all species in both light treatments. Blue dots are observed values for individual plants. (E) are exotic vines, (N) are native vines.

light treatment. *Delairea odorata* and *I. cairica* had the two highest predicted mean SLA in both treatments (Fig. 4, Table 1). *Cardiospermum grandiflorum* and *A. cordifolia* had similar SLA in the medium light, though *A. cordifolia* had much higher SLA in the low light (Fig. 4, Table 1).

Discussion

All species grew at faster rates under the higher light conditions that are reflective of rainforest edges, suggesting that quick invasion was most likely from disturbed edges or light gaps for all species. Low light conditions slowed growth, but the reduction in growth varied amongst species reflecting a differential risk of invasion and establishment into the rainforest interior. The two common native species had very low growth rates in all light levels, but particularly in the low light. They also showed a limited capacity to vary SLA and improve light capture relative to the invasive species. This suggests they would most likely establish in edges and better lit areas. This is despite one of these species being considered problematic; *Cissus antartica* can significantly smother vegetation along rainforest edges. Our results suggest that this issue will not occur under the canopy.

All exotic invasive species had higher relative growth rates than native species. Relative growth rate and high SLA are correlated with invasiveness (Poorter and Bongers 2006; Leishman et al. 2007; Van Kleunen et al. 2010; Dawson et al. 2011). However, some of the same species were used in Osunkoya et al. (2010) which measured no difference in RGR amongst native and exotic species in the tropics. Our work suggests that, at least in temperate areas, high relative growth rates were a characteristic of our invasive species.

Compared to self-supporting woody plants, the native vines in this study still sit on the “faster” side of the life history spectrum. For example, in a comparison of co-existing tropical trees and vines, Mello et al. (2020) found that the mean SLA for trees was $141 \text{ cm}^2/\text{g}$ ($\pm 62.41 \text{ SD}$) and vines was $177 \text{ cm}^2/\text{g}$ ($\pm 80.77 \text{ SD}$). In our study, the mean SLA for *C. antarctica* ($190 \text{ cm}^2/\text{g}$) and *P. pandorana* ($282 \text{ cm}^2/\text{g}$) grown in medium light was higher than that of the vines measured by Mello et al. (2020). Nevertheless, the difference in SLA between the exotic invasive and native species in this study may be due to natives investing more into leaf defence from herbivores leading to lower SLA, leaving the exotic invasives at an advantage with fewer herbivores in their introduced range (Keane and Crawley 2002).

Coupled with the higher growth rates measured, all exotic invasive vine species showed flexibility in leaf SLA when grown in low light conditions. All had higher SLA values under low light and were higher than both native species in all light conditions. Interestingly, the means in low light were associated with a great deal of variability amongst individuals (large 95% confidence intervals). Invasive exotic species, therefore, show plasticity in their responses to low light conditions, increasing the size of their leaves relative to the leaf biomass to increase light capture capacity. As a result, in comparison to the native species, all exotic invasive species will be capable of better

light harvesting under rainforest canopies, increasing photosynthesis and growth rates. Furthermore, increased SLA, even under medium light conditions, may also help them in forest gaps as they grow leaves better suited to the light environment they are in and, therefore, may be able to respond to canopy disturbances better (Liu et al. 2016b). Phenotypic plasticity of leaves is a trait often observed in invasives (Daehler 2003; Richards et al. 2006; Davidson et al. 2011) and Osunkoya et al. (2014) also found that leaf plasticity of exotic invasive vine species (including *A. cordifolia* and *A. sericifera*) was higher than phylogenetically similar native vine species. Therefore, while more native and exotic vine species need to be compared, it is likely that higher plasticity in leaves correlates with invasiveness and explains why exotic invasive vines often dominate in disturbed forests with patchy canopy cover, but may also increase their capability to invade low light less-disturbed closed forests.

We identified three growth strategies amongst the exotic invasive vines that we investigated, with regards to their risk to rainforest communities. These strategies may be a more general approach for other species, but further species would need to be considered to establish such strategies. Thus our descriptions of a strategy highlight some of the differences in growth responses of the exotic species we tested which may increase risk of invasion. The first strategy was associated with fast growth, exemplified by *D. odorata* and *I. cairica* which showed high SLAs and high mean stem growth rates under both canopy and edge conditions. Having fast stem growth rates and high stem biomass allocation is beneficial for vines as it allows individuals to compete with others through early access to canopy light (Falster and Westoby 2003; Llorens and Leishman 2008). High SLA provides leaves with more efficient energy capture to invest into growth (Poorter and Bongers 2006), although it also indicates that these species are likely to be short-lived (Westoby et al. 2004). *D. odorata* also invested very little into roots and did not produce woody stems suggesting a quick strategy with low competitive capacity. However, both of these species grow horizontal running stems which can form roots and support new stems, perhaps advantageous in capturing more area quickly under higher light conditions. This allows them to spread quickly and persist despite leaves (and perhaps stems) being replaced regularly. These species will be quick to establish both under canopy and at edges, but are likely to be more successful in forest gaps and edges and may do poorly through time under the canopy.

A second, more long-term invasion strategy was evident in the three other exotic species. These three had the highest relative growth rates under low light conditions, providing opportunities for invasion even within undisturbed rainforest patches; *Anredera cordifolia*, *Araujia sericifera* and *Cardiospermum grandiflorum*. While some *A. sericifera* individuals may be particularly effective at growing under the canopy, we also recorded some mortality in low light conditions. Surviving individuals produced few leaves, focusing resources on roots and stems suggesting a response to reach the canopy as quickly as possible. High dispersal capability using large numbers of wind-blown seeds (Vivian-Smith and Panetta 2005), coupled with high RGR in higher light, explains why *A. sericifera* is listed as a major weed in multiple countries (Vivian-Smith and Panetta 2005), where it damages vegetation and restricts regeneration, as well

being poisonous to humans and livestock (NSW DPI 2020). Germination has been shown to be significantly reduced in cool ($< 20\text{ }^{\circ}\text{C}$) and dark conditions (Vivian-Smith and Panetta 2005), suggesting that the maintenance of canopy cover can decrease establishment. Our results suggest that if it can establish under canopy, then it may well persist increasing its risk in rainforest interiors. If given the right conditions, *A. sericifera* is likely the species to build biomass fastest out of the species grown in this study and presents one of the highest invasion risks under rainforest canopies.

One other invasive species in this group of potential understorey invaders was *Anredera cordifolia*. This species was also able to have quite high RGRs under low light increasing its allocation to leaves and stems in this environment, compared to the location at the edges of rainforests. It also had much greater flexibility in changing leaf light capture under the canopy, compared to *A. sericifera*. At edges in ideal conditions, it can maximise growth rates through having amphistomatous leaves and high numbers of stomata (Boyne et al. 2013), increasing photosynthetic ability and efficiency. *A. cordifolia*, however, utilises an additional strategy. Under better growing conditions, resources are used to develop canopy-held tubers which are a robust dispersal strategy, providing new vegetative recruits which receive greater resources maternally than germinants. The strategy enables the species to persist through inclement conditions (Vivian-Smith et al. 2007). In the six months of this experiment, individuals in the medium light produced an average of 45 aerial tubers, highlighting the high propagule pressure of this species, as well as the speed in which it can reach reproductive maturity, which is a trait often used to explain invasiveness and feasibility of control (Panetta and Grigg 2021). Therefore, *A. cordifolia* can persist under canopies through reallocation of resources to leaves and stems and can spread throughout a site, particularly from the edges, making it difficult to eradicate and contain (Panetta and Grigg 2021). Tubers (included within the root component) resulted in lower allocation to stem growth, but provided a capacity to persist at the edges and create new individuals increasing invasion and risk. This places this species as one of the highest risk species under rainforest canopies.

Conclusion

Our work measured important growth parameters for a range of exotic vines and we were able to identify different strategies that influence how invasive exotic vine species may invade rainforests. While all species perform better in higher light conditions reflective of rainforest edges and gaps, the capacity to maintain higher levels of growth under rainforest canopies showed that some species may well establish and persist causing host tree damage within the rainforest. Coupled with vegetative growth strategies, we identified differences in the capacity of species to establish and persist under canopies. We suggest that maintaining canopy health and controlling edges, still remains the key tool for reducing vine invasion, although at least two species, *Araujia sericifera* and *Anredera cordifolia* have characteristics that suggest that directed control within rainforests is needed.

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Supplementary material 1

Difference in mean and 95% bounds, as well as the percentage overlap of mean values reported as a probability (Probability higher) between species for the physiological measurements taken

Authors: Adam Bernich, Kris French, Michael Bedward

Data type: xlsx

Explanation note: Species are compared within light treatments only. Probability higher reflects the values of the Species 1 being higher than Species 2. Asterisks in Species 1 column denotes exotic species.

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Link: <https://doi.org/10.3897/neobiota.90.110659.suppl1>

Supplementary material 2

Difference in mean and 95% bounds, as well as the percentage overlap of mean values reported as a probability (Probability higher than LL) for the seven species grown in the study

Authors: Adam Bernich, Kris French, Michael Bedward

Data type: xlsx

Explanation note: Asterisks denote exotic species.

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Supplementary material 3

The dry weight of the individuals used as the initial values to calculate relative growth rates for each species

Authors: Adam Bernich, Kris French, Michael Bedward

Data type: xlsx

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Root hemiparasites suppress invasive alien clonal plants: evidence from a cultivation experiment

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Abstract

Alien invasive plants threaten biodiversity by rapid spread and competitive exclusion of native plant species. Especially, tall clonal invasives can rapidly attain strong dominance in vegetation. Root-hemiparasitic plants are known to suppress the growth of clonal plants by the uptake of resources from their below-ground organs and reduce their abundance. However, root-hemiparasites' ability to interact with alien clonal plants has not yet been tested.

We explored the interactions between native root-hemiparasitic species, *Melampyrum arvense* and *Rhinanthus alectorolophus* and invasive aliens, *Solidago gigantea* and *Symphyotrichum lanceolatum*. We investigated the haustorial connections and conducted a pot experiment. We used seeds from wild hemiparasite populations and those cultivated in monostands of the invasive plants to identify a possible selection of lineages with increased compatibility with these alien hosts. The hemiparasitic species significantly suppressed the growth of the invasive plants. *Melampyrum* inflicted the most substantial growth reduction on *Solidago* (78%), followed by *Rhinanthus* (49%). Both hemiparasitic species reduced *Symphyotrichum* biomass by one-third. Additionally, *Melampyrum* reduced the shoot density of both host species. We also observed some transgenerational effects possibly facilitating the growth of hemiparasites sourced from subpopulations experienced with the host.

Native root hemiparasites can effectively decrease alien clonal plants' biomass production and shoot density. The outcomes of these interactions are species-specific and may be associated with the level of clonal integration of the hosts. The putative selection of lineages with higher performance when attached to the invasive novel hosts may increase hemiparasites' efficiency in future biocontrol applications.

Keywords

Asteraceae, biological invasion, biotic resistance, Orobanchaceae, physiological integration, pot experiment, restoration

Introduction

Alien plant invasions represent a component of global change with profound effects on diversity, ecosystem functioning and services. Invasive species broadly vary in their specific impacts on the habitats they invade due to different abilities to spread and achieve dominance or mechanisms of interaction with native biota (Blackburn et al. 2014). Of particular concern are the so-called transformer invaders (Richardson et al. 2000), which can invade indigenous natural communities over large areas, attain high dominance and change ecosystem functioning. Alien tall clonal herbs with below-ground rhizomes are frequent examples of transformer invaders in grasslands due to their increased competitiveness leading to the exclusion of native plants from infested vegetation throughout temperate regions (Divíšek et al. 2018; Wang et al. 2019; Wan et al. 2021; Lanta et al. 2022). Although conventional control management, represented by mowing or grazing, can reduce the density of the invasive clonal herbs to some extent (e.g. Nagy et al. (2020); Szymura et al. (2022)), they are usually not eliminated and may spread rapidly from the rhizomes if the management measures are ceased. More drastic restoration measures (e.g. use of herbicides, long-term shading) may eradicate the invaders, but can also adversely affect native species, making their use problematic in areas with conservation value (e.g. Weber and Jakobs (2005); Szymura et al. (2022)).

Native parasitic plants have recently been suggested as potential biocontrol agents for a wide range of invasive plants globally (Těšitel et al. 2020). Following the biotic resistance hypothesis (Maron and Vilà 2001), generalist native adversaries, such as parasitic plants, may impede the success of invaders due to the lack of defence or tolerance mechanisms of the host plants against parasitism (Cameron and Seel 2007). Clonal hosts could be especially harmed by parasitism, as the parasitic uptake of resources targets the cornerstone of their growth strategy, that is, the spatial spread of vegetative ramets and clonal integration (e.g. Song et al. (2013); Roiloa (2019)), i.e. the transfer of resources amongst interconnected ramets via rhizome network, which facilitates efficient resource acquisition and sharing (Kavanová and Gloser 2005; Gao et al. 2021). However, parasitic plants may turn this advantage into a liability. A parasite that attaches to one ramet may access nutrients within the network, leading to its vigorous growth and potentially marked biomass decline of the clonal host, including the non-infected ramets (Lepš and Těšitel 2015; Gao et al. 2021). This could explain a substantial decrease in the clonal hosts' abundance in the communities with parasitic plants observed in several studies (Declerck et al. 2013; Demey et al. 2015; Somodi et al. 2018). Moreover, field experiments have demonstrated the ability of root-hemiparasitic *Rhinanthus* species to significantly reduce harmful expansive clonal grass *Calamagrostis epigejos* from semi-natural grasslands (Těšitel et al. 2017, 2018),

which was consequently introduced to ecological restoration practice (Lukavský 2020; SPPK D02 002 2021).

Amongst parasitic plants, species of root hemiparasites (or, more precisely, Euphytoid parasites in the new parasitic plant classification of Teixeira-Costa and Davis (2021)) appear to be particularly suitable candidates for suppressing clonal invasive species due to their low host specificity (e.g. Matthies (2017, 2021)), capacity to substantially suppress host growth (e.g. Press et al. (2005); Těšitel et al. (2015b); Matthies (2021)) and ability to form dense populations (van Hulst et al. 1987; Mudrák and Lepš 2010; Heer et al. 2018). Despite available evidence on the negative effects of root hemiparasites on expansive species (reviewed by Těšitel et al. (2020)), only one study has investigated the effect of a root-hemiparasitic species on an alien invader (Walder et al. 2019), which, however, did not show any adverse impact of the parasite on the host species. Two reasons may explain this lack of empirical research on interactions between root hemiparasites and alien invaders. First, hemiparasites and alien invaders may not share the same habitats. For instance, in Central Europe, an analysis of habitats of hemiparasitic species identified natural and semi-natural communities as their principal habitats (Těšitel et al. 2015a). These habitats are simultaneously characterised by low levels of alien invasions (Pyšek et al. 2012). Second, establishing a parasitic association with alien invaders may be difficult. Although hemiparasites are mostly host generalists, host quality (i.e. the extent of support of parasite growth) varies between species (e.g. Rowntree et al. (2014); Matthies (2017, 2021)). Native hemiparasitic species lack a common evolutionary history with non-indigenous plants. The lack of experience with an alien host may limit a hemiparasite's efficiency of resource withdrawal on the one hand, but also the host's resistance or tolerance to parasitism on the other, as predicted by the biotic resistance hypothesis. Compatibility with a host may also be affected by high intra- and interpopulation genotypic variability of the annual hemiparasites (Mutikainen et al. 2000; Rowntree et al. 2011; Unachukwu et al. 2017; Rowntree and Craig 2019; Moncalvillo and Matthies 2023). The recognised ability to rapidly evolve ecotypes adapted to various environmental conditions (Zopfi 1993; Pleines et al. 2013) may further facilitate the interaction with novel host species.

In this paper, we investigated the interactions between root-hemiparasitic *Rhinanthus alectorolophus* and *Melampyrum arvense* (Orobanchaceae) and the alien invasive clonal species *Solidago gigantea* and *Symphotrichum lanceolatum* (Asteraceae). First, we examined the anatomy of haustoria to determine whether the hemiparasites can form functional parasitic connections with the novel hosts. Second, we set up a comprehensive pot experiment to study the effect of host identity on hemiparasite performance and the impact of hemiparasite infection on the two hosts. We expected to identify differences in vitality (measured by biomass production) of the two hemiparasite species (hypothesis 1), which should be reflected by a difference in host suppression (hypothesis 2). Specifically, we expected lower host suppression by *Rhinanthus*, given its general preference for grass or legume hosts (Matthies 2021), than in *Melampyrum*, which has been shown to flourish when attached to various forbs, including many Asteraceae

(Matthies 2017). Furthermore, we investigated the potential selection of hemiparasite lineages and their effect on host–hemiparasite interactions. To do so, we used seeds from hemiparasites that had grown for two years in monoculture stands of the two host species and compared their performance to plants from the original population from a species-rich grassland ('naïve' plants), i.e. all tested seed sources per hemiparasite species originated from a single hemiparasite population. We hypothesised that growth in a host monoculture might lead to a selection of lineages better adapted to the given host, reflected by improved hemiparasite growth and possibly a more deleterious effect on that host (hypothesis 3).

Materials and methods

Study species

Melampyrum arvense L. and *Rhinanthus alectorolophus* (Scop.) Pollich are annual xylem-feeding root-hemiparasitic species native to Europe. *Melampyrum* typically grows in dry grasslands and steppes, while *Rhinanthus alectorolophus* (Scop.) Pollich favours dry to mesic grasslands. *Solidago gigantea* Aiton and *Symphyotrichum lanceolatum* (Willd.) G. L. Nesom are perennial rhizomatous species from the Asteraceae family, originating from North America (Pyšek et al. 2012). They began spreading across Europe in the 19th century and have become serious invaders (Weber and Jakobs 2005; Jedlička and Prach 2006; Axmanová et al. 2021). *Solidago* and *Symphyotrichum* are considered typical wetland species, but they also occur in disturbed anthropogenic habitats, poorly-managed fields, pastures and meadows within their native range (Chmielewski and Semple 2001; Weber and Jakobs 2005). *Solidago* has a broader ecological niche in the invaded areas, also occupying drier and nutrient-poorer soils (Weber and Jakobs 2005). Both species have a perennial rhizome, which, in the spring, produces a cohort of shoots that start to flower in late summer (*Solidago*) or early autumn (*Symphyotrichum*) and yield numerous tiny wind-dispersed seeds. Jedlička and Prach (2006) noted the high viability of *Symphyotrichum lanceolatum* seeds, which, combined with the effective ability to penetrate established vegetation, triggers the high invasive potential of this species.

Haustorial connection

We initiated a pilot cultivation trial to examine the anatomy of haustorial connections between the hemiparasites and the two invasive hosts. The cultivation was set up in the experimental garden of the Department of Botany and Zoology at Masaryk University in Brno, Czech Republic. The hemiparasites' seeds were collected from species-rich vegetation in the summer of 2019 (see Suppl. material 1: appendix S1 for localisations). In the autumn of 2019, we transplanted rhizomes of host species into 15 × 15 × 20 cm pots (narrower at the bottom, corresponding to 3.6 litres), filled with a mixture

of peat and garden soil (ratio 1:3). In October, we sowed 20 hemiparasite seeds on each pot. We established five replicates for each hemiparasite-host combination.

In June 2020, we rinsed the hosts' roots, harvested the haustoria and preserved them in 70% ethanol. Following the method of Soukup and Tylová (2014), we dehydrated the samples, transferred them to anhydrous butanol, infiltrated and then embedded them in paraffin. We prepared 12 μm sections using a sliding microtome (Reichert, Wien, Austria) and de-waxed and stained them with phloroglucinol-HCl (Wiesner solution) (Liljegren 2010) to colour the lignified cell walls.

Cultivation experiment

We established the main pot experiment in autumn 2021 to investigate and quantify the outcome of the novel interactions for the hemiparasites and the extent of host suppression. For each hemiparasitic species, we used three seed sources: (i) seeds from a wild population growing in a species-rich grassland and (ii) seeds from plants originally obtained from the same populations as in (i), but which had been growing since 2019 in monostands of the two invasive host species. The aim was to investigate the potential selection of genotypes more adapted to the specific hosts. More specifically, the monostands were mown in the autumn of 2019, after which we sowed the hemiparasites' seeds. In 2020, the monostands with hemiparasites were mown in July and October. We collected ripe hemiparasite seeds from all populations from June to July 2021. The seeds were stored at room temperature before use. As both host species produce a dense rhizome network in the topsoil layer, we collected soil blocks with rhizomes from monostands of each host species to establish host cultivation in September 2021. First, we removed the above-ground biomass and then cut approx. 12 \times 12 cm rhizome blocks with a spade. The rhizomes were then inserted into the same pots and soil substrate described in the chapter 'Haustorial connection'. See Suppl. material 1: appendix S1 for GPS coordinates of the sites of hemiparasites' seed and host plants' origin.

The experimental design comprised: (i) an uninfected control treatment (host species without hemiparasite seed addition) and three types of 'infected' treatments (with hemiparasite seeds addition), i.e. treatments (ii) 'naïve' (seeds of hemiparasites originating from a wild population), (iii) 'home' (seeds from hemiparasites growing for two years in a monostand of a host species and then sown with the same host species in the pot) and (iv) 'cross' (seeds from hemiparasites growing for two years in a monostand of one host species and then sown into the pot with the other host species) (see the scheme of the origin of hemiparasites' seeds in Fig. 1). Both hemiparasites were sown with both invasive species, resulting in 14 treatments. Each treatment consisted of 10 replicates of the pots, totalling 140 pots. Each pot in the 'infected' treatments (treatments ii–iv) received 40 seeds of one of the hemiparasitic species. Seeds were spread on the surface and gently mixed with the topsoil layer. The pots were then placed in the experimental garden in Brno, following a completely randomised design and irrigated. During spring 2022, the pots were irrigated as necessary. In April 2022, seedlings of

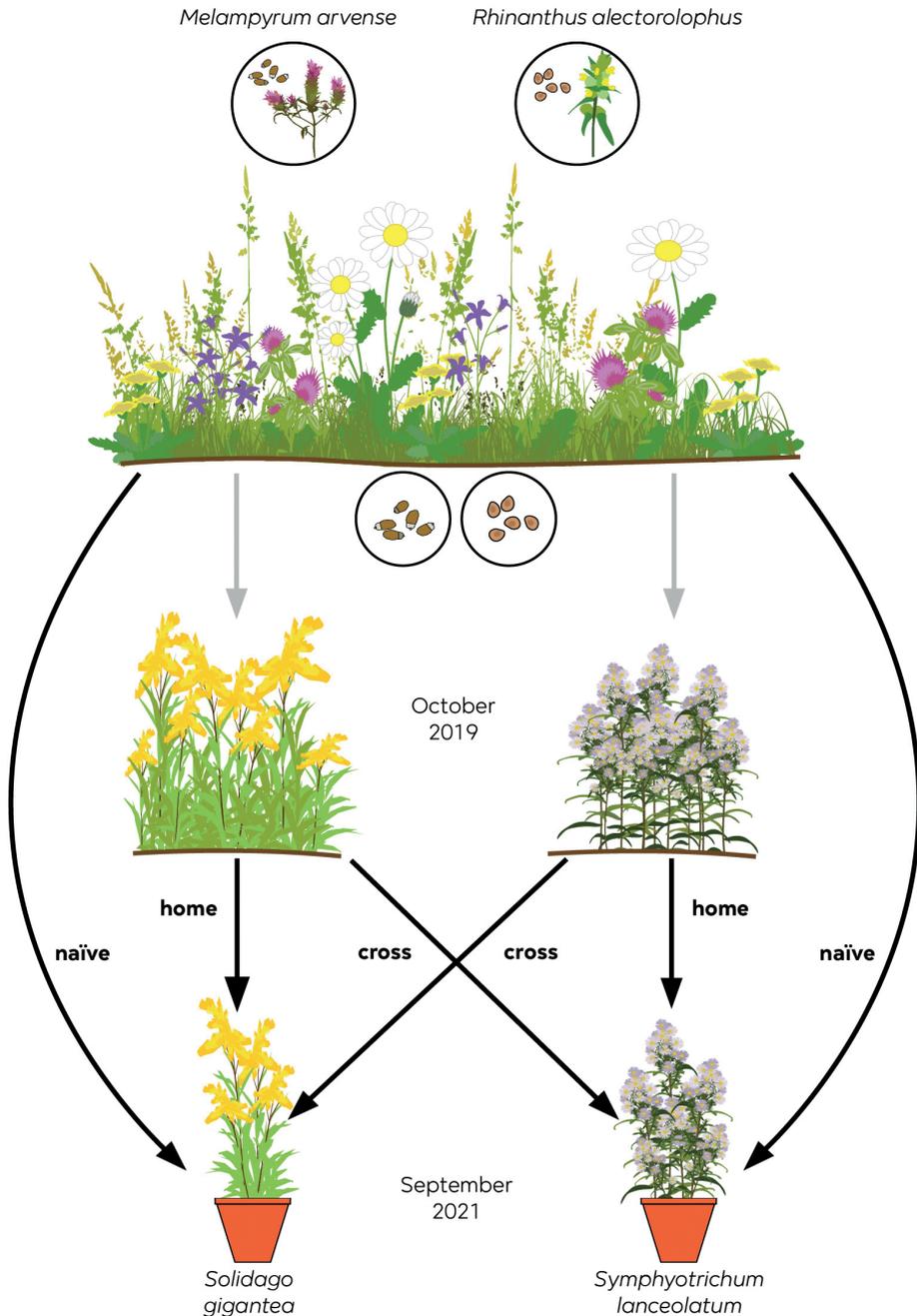


Figure 1. Scheme of origin of the hemiparasites' seeds used in the cultivation experiment. In October 2019, seeds of *Melampyrum arvense* and *Rhinanthus alectorolophus* from a single population per species, originating from a species-rich grassland, were sown in monostands of the host species *Solidago gigantea* and *Symphyotrichum lanceolatum*. By 2021, hemiparasite seeds collected from the host species' monostands and the original hemiparasite population were used in the cultivation experiment resulting in three types of hemiparasite seed sources: 'naïve', 'home' and 'cross'.

non-target species were removed from the pots. The pots were spaced 30 cm apart and their position within the experimental matrix was changed three times before harvest at the beginning of June 2022.

The experiment was harvested during hemiparasite flowering. We cut the above-ground biomass and counted the number of host shoots and hemiparasitic plants that survived in each pot. The hemiparasite and host biomass from each pot were dried separately at 80 °C and weighed. Schmid et al. (1995) revealed a strong dependency of sexual reproduction and clonal growth on plant size as well as a threshold size for sexual reproduction in *Symphytotrichum lanceolatum* and *Solidago canadensis*, a species closely related to *Solidago gigantea*. We thus expected the vegetative biomass to reflect host fitness and reproductive potential sufficiently.

Statistical analyses

Initially, we conducted an exploratory analysis of patterns in counts of hemiparasite individuals, host ramets and above-ground biomass production to identify pots that were not representative due to insufficient host or hemiparasite recruitment. Only pots with at least six host shoots and three hemiparasite individuals (in infected treatments) were subsequently included in the analysis ($n = 132$ out of 140 pots). Scatterplots of biomass vs. individual or shoot counts (Suppl. material 1: appendices S2, S3) demonstrated low correlations, indicating compensatory growth in pots retained for the analysis.

We used linear models to analyse the following parameters: hemiparasite above-ground biomass, the number of individuals, mean biomass per individual and host above-ground biomass, the number of shoots and mean biomass per shoot. All variables were log-transformed before analysis to improve the normality of residuals and homogeneity of variances. The analysis of each parameter, used as response variables, was conducted at two levels: (i) the species-level model included hemiparasite, host species and their interaction as predictors. Seed-source treatments were disregarded in this analysis; (ii) seed-source analysis consisted of a series of linear models, one for each host–hemiparasite combination, with seed-source treatment as a single predictor. In this analysis, we set treatment contrasts with the ‘naïve’ treatment as the baseline level, to which the two other treatments were compared. Only biomass data were tested in the seed-source level analysis.

We first built a saturated model for each analysis with all candidate predictors and interactions. Individual terms of the saturated models were tested by an F-test, the results of which are reported in ANOVA tables as in a classical two-way ANOVA with interactions. Non-significant ($P > 0.05$) terms were subsequently removed from the models in the backward predictor selection procedure. Non-significant main effects were retained if a predictor was involved in a significant interaction. The resulting minimal adequate models were then used to extract regression coefficients and their associated tests of significance. This approach was allowed by the nature of our data coming from a manipulative experiment with a balanced design, which implies the or-

thogonality of the predictors. We acknowledge that the orthogonality was not perfect because we removed a few pots with low establishment of hosts or parasites. Still, the collinearity between the tested effects (host and parasite predictors) was minimal as measured by the phi-coefficient ($\varphi = 0.026$; $\chi_1 = 0.0084$; $P = 0.927$), which justifies the validity of the interaction-term testing and supports backward selection as a suitable model-selection approach. All analyses were performed in R, version 4.2.2 (R Core Team 2022).

Results

Functional haustorial connection

Both hemiparasitic species formed fully developed haustoria on the roots and rhizomes of both host species. In all cases, the xylem bridge from hemiparasite haustoria reached the xylem vessels of the hosts. No signs of a defensive reaction by the hosts were observed (Fig. 2).

Host–Hemiparasite interaction on the species level

Hosts successfully resprouted from rhizomes in the transferred soil blocks; only four pots had to be omitted because of insufficient sprouting (Fig. 3, Suppl. material 1: appendix S4). The number of hemiparasite plants varied in the pots, but their establishment was generally successful, with only four pots omitted from the experiment due to poor hemiparasite establishment. On average, 10.9 *Melampyrum* plants were harvested in pots with both host species (max. 20 individuals). In contrast, significantly higher average numbers of *Rhinanthus* plants, 16.2 and 13.2, were harvested in pots with *Solidago* and *Symphyotrichum* (max. 23 individuals), respectively (Table 1, Fig. 4, Suppl. material 2 for the primary data). Hemiparasite biomass production differed between the two species and was also significantly affected by the host identity (Table 1). Specifically, *Melampyrum* grew larger than *Rhinanthus* ($t_{110} = 11.25$, $P < 10^{-6}$) and *Solidago* supported a more vigorous hemiparasite growth than *Symphyotrichum* ($t_{110} = 10.12$, $P < 10^{-6}$). These effects were additive, i.e. the difference in the host quality had a similar impact on both hemiparasitic species (Fig. 4). Similar trends and significant interactions were also found concerning the average biomass of hemiparasite individuals (Table 1). *Melampyrum* individuals were consistently larger than *Rhinanthus* and both hemiparasitic species produced larger specimens on *Solidago* than on *Symphyotrichum*. However, this trend was less pronounced in *Rhinanthus*, i.e. *Rhinanthus* individuals growing with *Symphyotrichum* were larger than expected by additive effects ($t_{109} = 2.57$, $P = 0.012$; Fig. 4).

Regarding host suppression, we identified strong interactive effects of host and hemiparasite species identities on the host biomass (Table 1). The hemiparasitic spe-

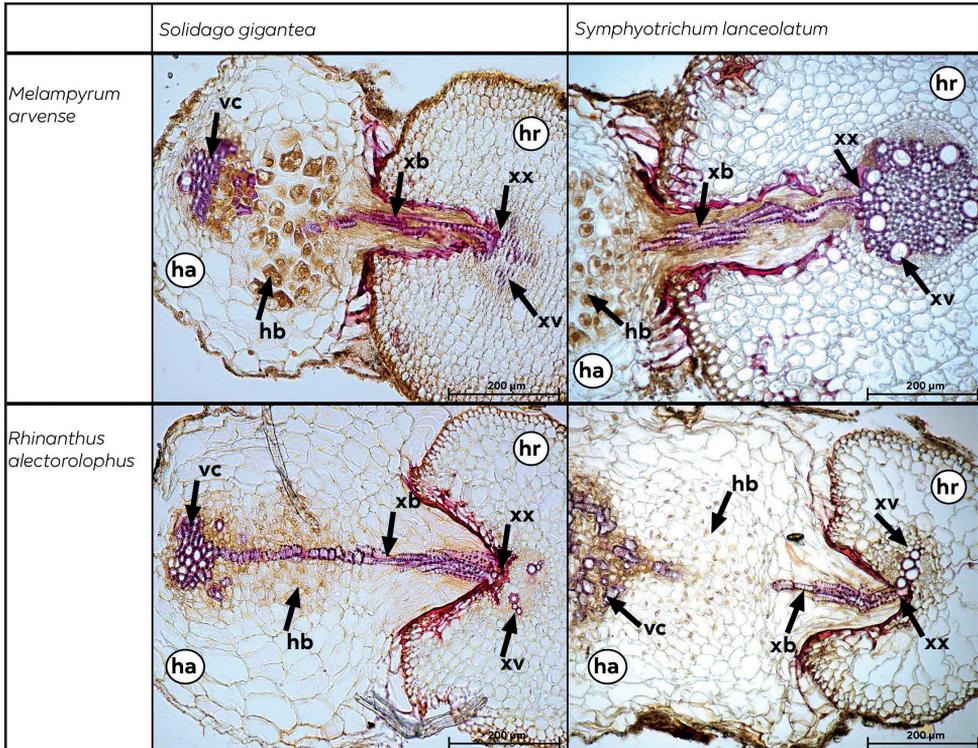


Figure 2. Cross sections of haustorial connections between two root-hemiparasitic species and their hosts. In the hemiparasite haustoria (ha), there is a hyaline body (hb), the vascular core of the haustorium (vc) and a xylem bridge (xb) leading to host xylem vessels (xv) in the host root (hr); xx – xylem–xylem contact.

cies significantly reduced host biomass relative to uninfected controls (*Melampyrum*: $t_{126} = -10.1$, $P < 10^{-6}$, *Rhinanthus*: $t_{126} = -4.53$, $P < 10^{-4}$), but the suppression was significantly more pronounced in *Solidago* infected by *Melampyrum* ($t_{126} = 4.50$, $P < 10^{-5}$; Fig. 5). Overall, *Solidago* biomass was reduced by 77.6% and 49.1% on average when infected by *Melampyrum* and *Rhinanthus*, respectively. *Symphyotrichum* biomass was reduced by 31.6% and 35.2% on average by *Melampyrum* and *Rhinanthus*, respectively. Host biomass was reduced by decreasing the number of host shoots or reducing the average biomass of host shoots. While *Melampyrum* acted in both ways, *Rhinanthus* mainly decreased the average host shoot biomass (Fig. 5). In detail, *Melampyrum* reduced the number of host shoots per pot ($t_{128} = -4.05$, $P < 10^{-4}$) by 33% in *Solidago* and 21% in *Symphyotrichum*. The effect of *Rhinanthus* on the host shoot number was not significant ($t_{128} = -0.76$, $P = 0.45$). Both *Melampyrum* ($t_{126} = -7.07$, $P < 10^{-6}$) and *Rhinanthus* ($t_{126} = -4.00$, $P < 0.001$) reduced the average biomass of host shoots. While *Rhinanthus* reduced the average shoot biomass of both hosts to a similar extent, *Melampyrum* was significantly less deleterious to *Symphyotrichum* than to *Solidago* ($t_{126} = 3.98$, $P < 0.001$).

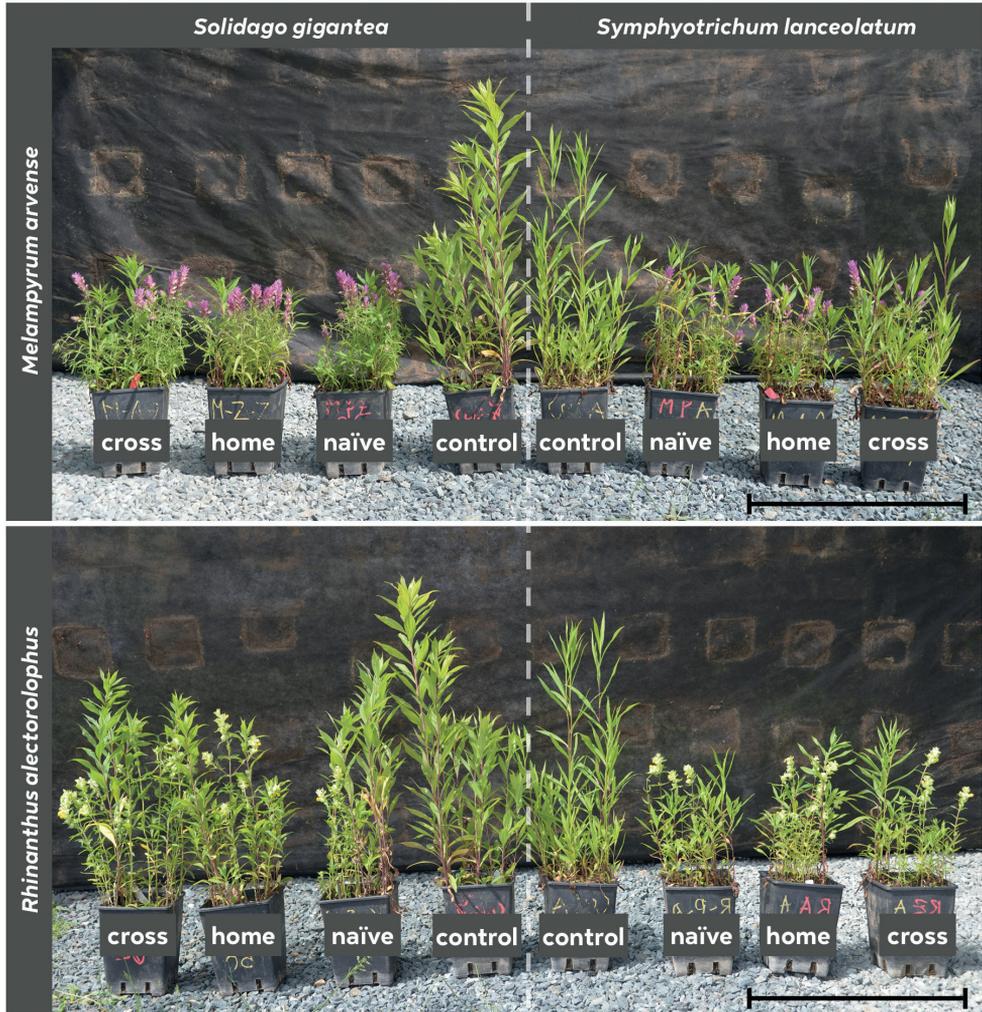


Figure 3. Representative pots for each hemiparasite seed-source treatment ('cross', 'home', 'naïve') and the uninfected control. *Solidago gigantea* (left) and *Symphyotrichum lanceolatum* (right) are infected by *Melampyrum arvense* (top) or *Rhinanthus alectorolophus* (bottom). The bottom photo is flipped vertically for clarity of the experiment presentation. Photographic documentation of all experimental pots is provided in Suppl. material 1: appendix S4. Scale bars: 50 cm.

Effect of the hemiparasite seed origin on the interaction

We identified the significant effects of the hemiparasite seed-source treatments on some interactions. Total hemiparasite biomass was affected in the case of *Melampyrum* growing on *Solidago* ($R^2 = 0.29$, $F_{2,24} = 4.98$, $P = 0.016$) and *Rhinanthus* growing on *Symphyotrichum* ($R^2 = 0.32$, $F_{2,25} = 5.81$, $P = 0.008$). Specifically, *Melampyrum* plants

Table 1. Analysis of variance tables summarising the effects of hemiparasite and host species identity on the growth of hemiparasites and hosts.

Response	Effect	df	Sum Sq.	F	P
Hemiparasite biomass	Hemiparasite	1	13.72	121.95	< 10^{-6}
	Host	1	11.65	103.48	< 10^{-6}
	Hemiparasite \times Host	1	0.24	2.10	0.15
	Residuals	109	12.28		
Hemiparasite count per pot	Hemiparasite	1	3.89	19.64	< 10^{-4}
	Host	1	0.35	1.75	0.19
	Hemiparasite \times Host	1	0.29	1.46	0.23
	Residuals	109	21.60		
Hemiparasite average biomass	Hemiparasite	1	32.23	203.10	< 10^{-6}
	Host	1	7.97	50.22	< 10^{-6}
	Hemiparasite \times Host	1	1.05	6.61	0.011
	Residuals	109	17.30		
Host biomass	Hemiparasite*	2	16.11	46.12	< 10^{-6}
	Host	1	0.02	0.13	0.72
	Hemiparasite \times Host	2	7.69	22.00	< 10^{-6}
	Residuals	126	22.00		
Host shoot count per pot	Hemiparasite*	2	2.53	13.09	< 10^{-5}
	Host	1	5.07	52.42	< 10^{-6}
	Hemiparasite \times Host	2	0.31	1.59	0.21
	Residuals	126	12.18		
Host shoot average biomass	Hemiparasite*	2	7.28	18.35	< 10^{-6}
	Host	1	5.77	29.10	< 10^{-6}
	Hemiparasite \times Host	2	5.04	12.71	< 10^{-5}
	Residuals	126	24.99		

*The hemiparasite effect on host biomass also comprises non-infected control as an extra level.

in the 'cross' treatment (seeds from plants previously grown with the alternative invasive host) produced significantly less biomass ($t_{24} = -2.80$, $P = 0.010$) compared to the 'naïve' treatment (seeds from species-rich vegetation), while the biomass of *Melampyrum* on *Solidago* from the 'home' (seeds from plants previously grown with the same host species) and 'naïve' treatment did not significantly differ (Fig. 6). Conversely, the biomass of *Rhinanthus* on *Symphyotrichum* was significantly higher in the 'home' treatment compared to the 'naïve' treatment ($t_{25} = 3.09$, $P = 0.005$) and the hemiparasite biomass in the 'cross' and 'naïve' treatment did not differ (Fig. 6).

Host biomass was significantly affected only in the case of *Solidago* infected by *Rhinanthus* ($R^2 = 0.27$, $F_{2,27} = 5.09$, $P = 0.013$) (Fig. 7). Here, *Rhinanthus* of 'home' and 'cross' treatments suppressed *Solidago* biomass more than 'naïve' *Rhinanthus* plants ($t_{27} = -2.73$, $P = 0.011$ and $t_{27} = -2.80$, $P = 0.009$ for 'home' and 'cross' treatments, respectively).

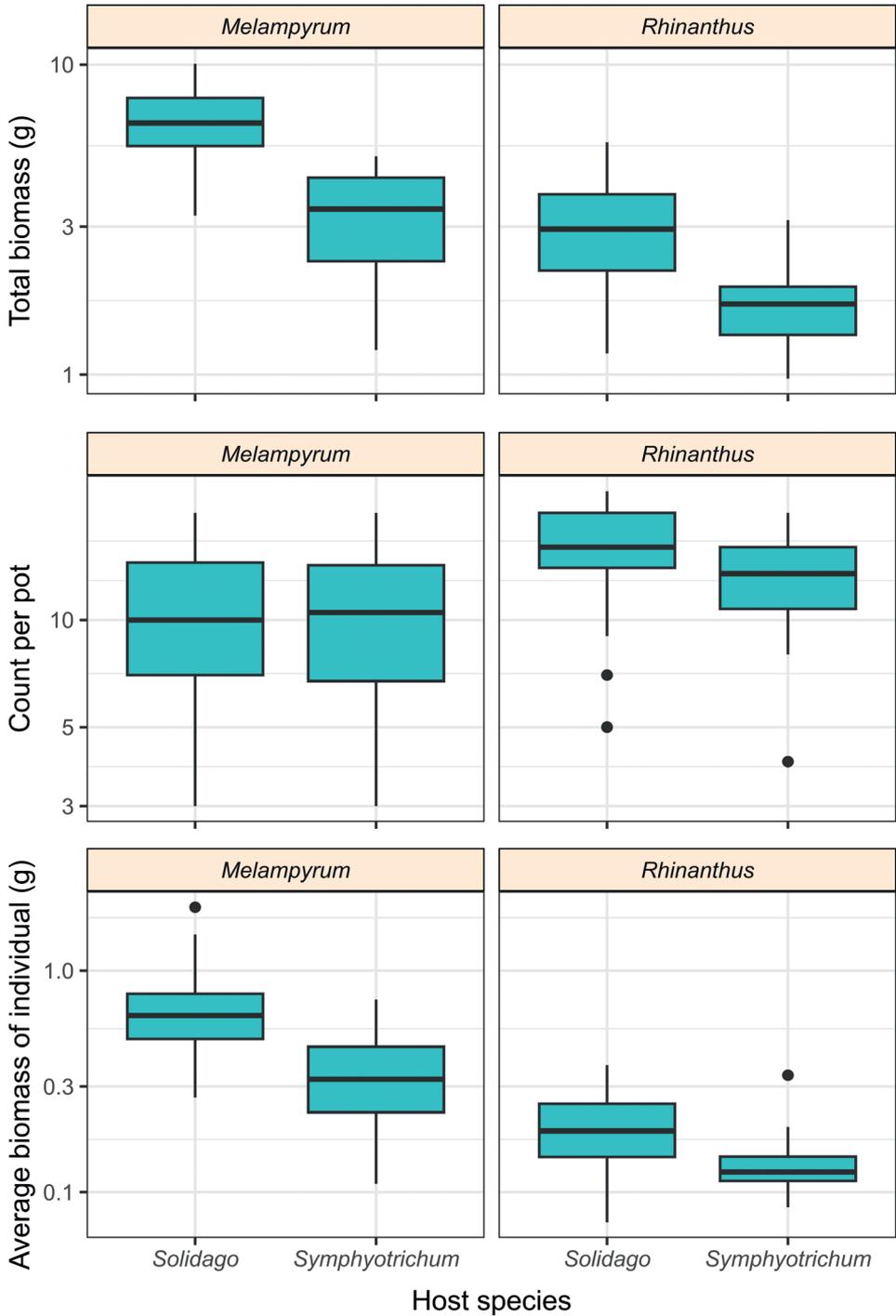


Figure 4. Effects of host species on the total biomass, number of individuals per pot and average biomass of the individuals of the two hemiparasitic species. Boxplots represent median, quartiles and ranges. See Table 1 for the ANOVA tables summarising significance tests. Note the logarithmic scale of the y-axes.

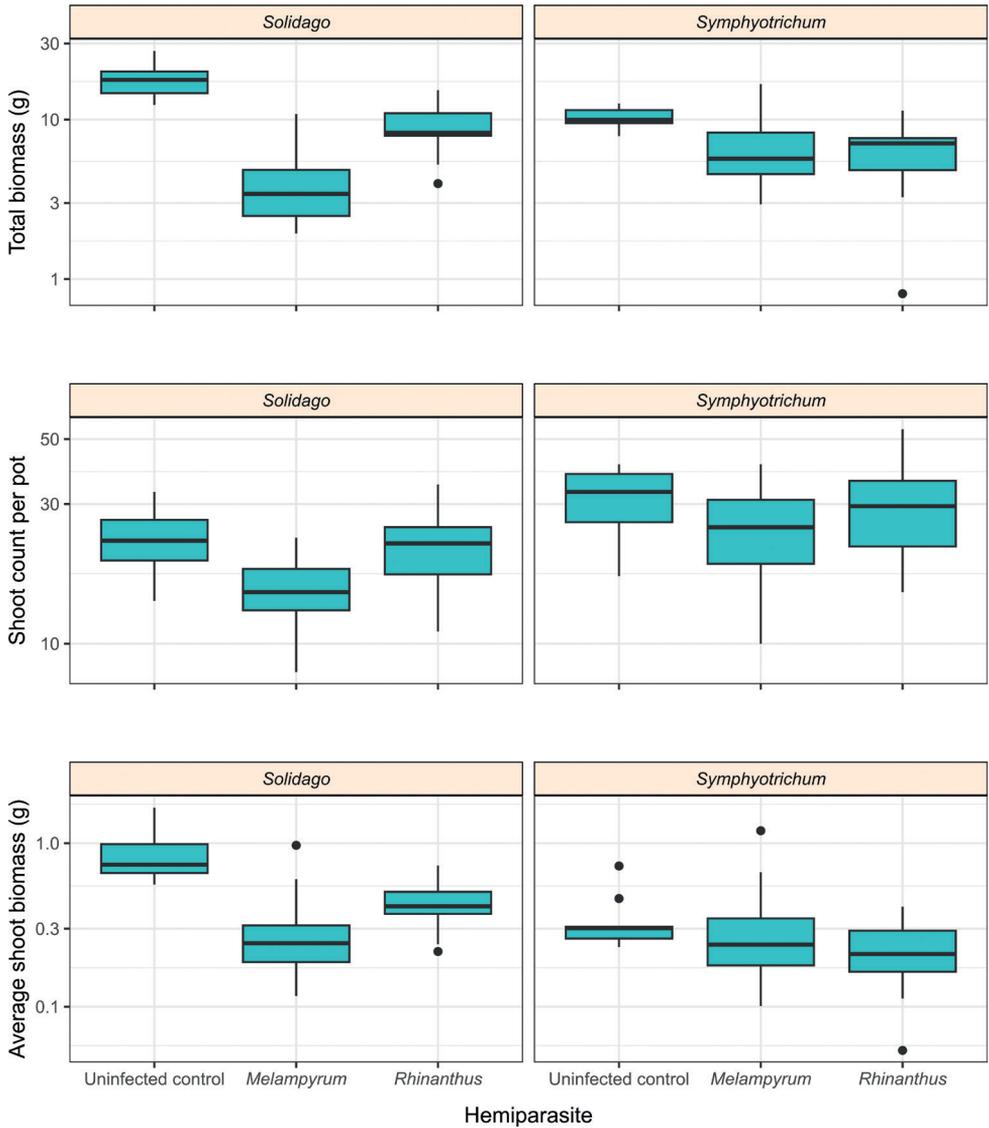


Figure 5. Effect of hemiparasite infection on total biomass, number of shoots per pot and average shoot biomass of the two host species. Boxplots represent median, quartiles and non-outlier ranges, with outliers displayed as points outside the non-outlier ranges. Note the logarithmic scale of the y-axes. See Table 1 for the ANOVA tables summarising significance tests.

Discussion

The outcome of the novel host–hemiparasite interactions

Both root-hemiparasitic species established a functional parasitic association with the two novel host species, as evidenced by functional haustorial connection, vital growth

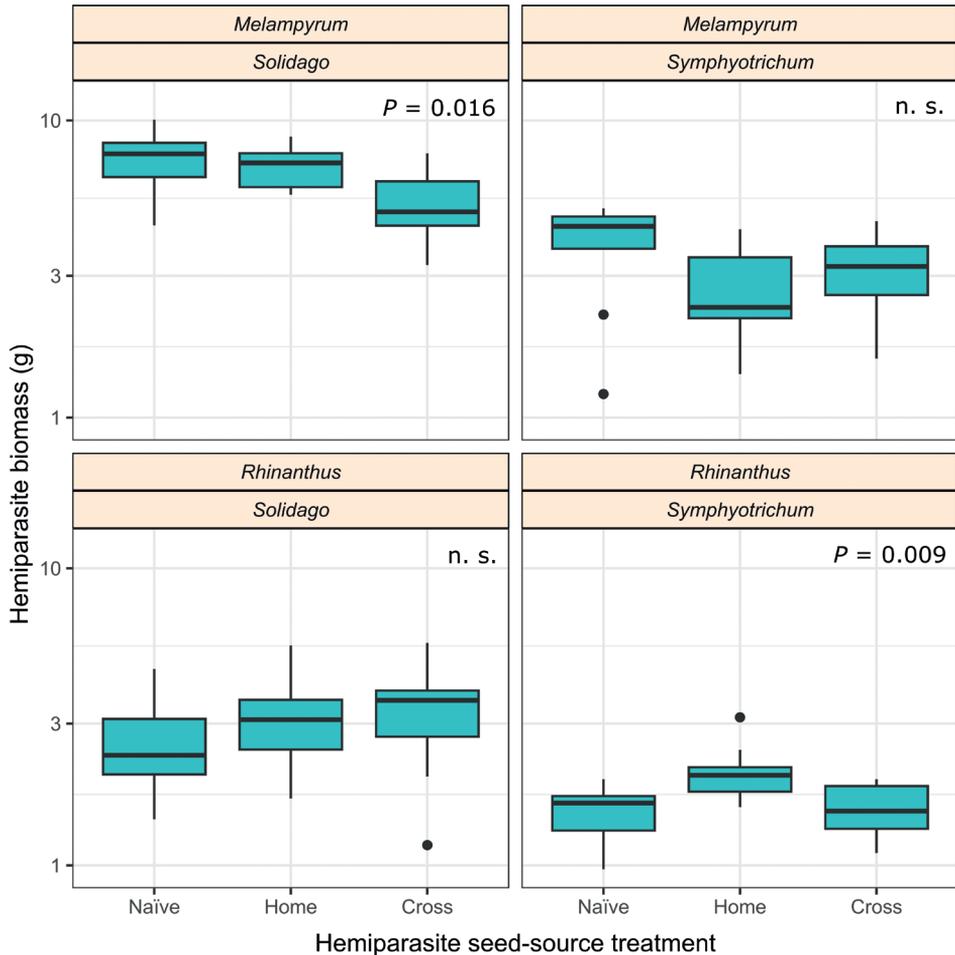


Figure 6. Effect of seed-source treatments on hemiparasite biomass production categorised by the individual host–hemiparasite combinations. Boxplots represent median, quartiles and non-outlier ranges, with outliers displayed as points outside the non-outlier ranges. P -values indicate significant effects of seed-source treatments. Note the logarithmic scale of the y-axes.

and flowering of both parasites (Figs 2, 3). In line with our first hypothesis, the hemiparasite species differed in compatibility with the two invasive hosts from Asteraceae, with *Melampyrum* proving a more efficient parasite than *Rhinanthus*. This outcome is not surprising, as *Melampyrum* has previously been shown to thrive when attached to a series of forbs. Asteraceae species, such as *Achillea millefolium*, *Matricaria chamomilla* and *Taraxacum officinale*, were even amongst the top five hosts out of 27 potential hosts tested (Matthies 2017). The average biomass of *Melampyrum* individuals of ca. 500 mg on *Solidago* and 300 mg on *Symphyotrichum* classifies these species amongst the best or moderately good hosts, respectively (in comparison to Matthies (2017)). *Rhinanthus* spp. have been repeatedly reported to grow better when attached to grasses or legumes

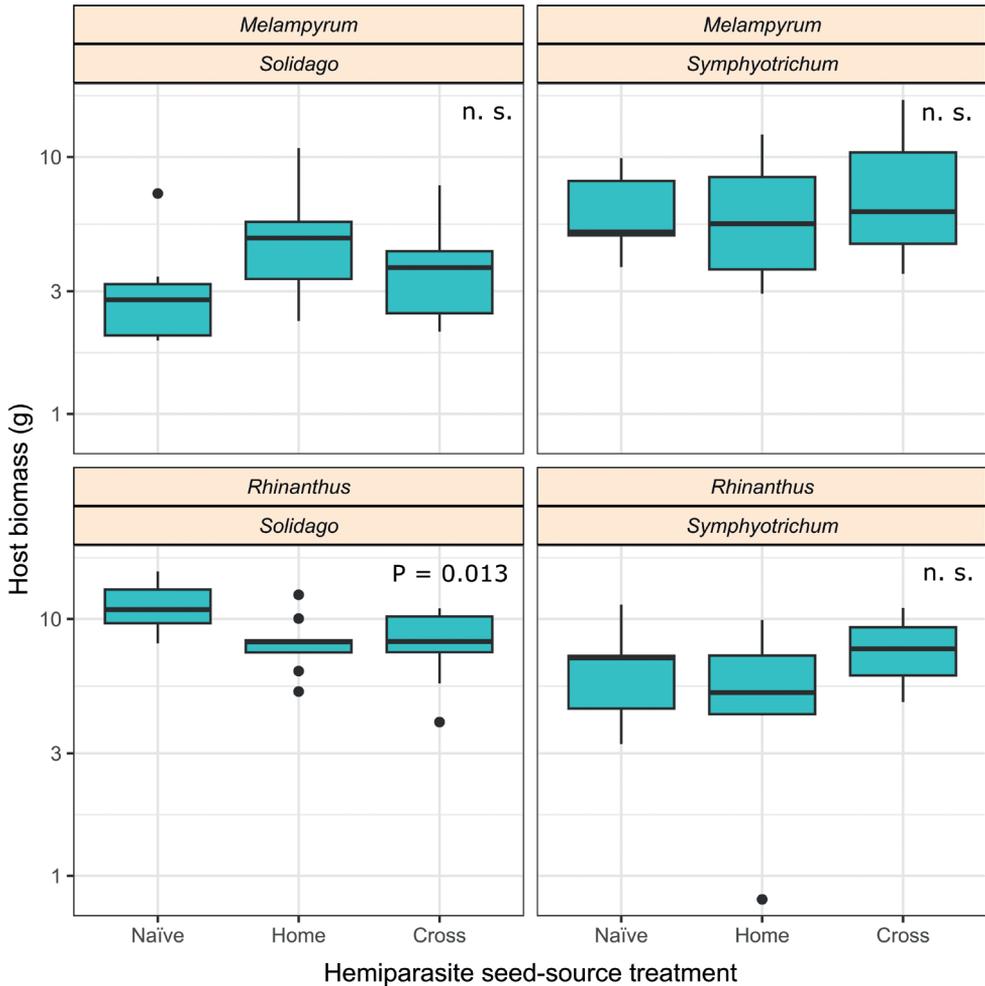


Figure 7. Effect of seed-source treatments on host biomass production in infected pots categorised by the individual host–hemiparasite combinations. Boxplots represent median, quartiles and non-outlier ranges, with outliers displayed as points outside the non-outlier ranges. *P*-values indicate significant effects of seed-source treatments. Note the logarithmic scale of the y-axes.

than forbs (Cameron and Seel 2007; Rowntree et al. 2014; Matthies 2021). The biomass production of *Rhinanthus alectorolophus*, converted to values per 1 m², amounted to 136 g DW and 75.6 g DW when attached to *Solidago* and *Symphyotrichum*, respectively. These values are lower than those reported for the best grass hosts in a recent field cultivation experiment (Hejduk et al. 2020). Still, *Solidago* can be considered a similarly good host for *Rhinanthus alectorolophus* as *Lotus corniculatus*, the best host amongst legumes. *Symphyotrichum* is a host of lower quality, but still comparable to some grasses (*Festuca rubra*) or legumes (*Trifolium hybridum*) (Hejduk et al. 2020). Compared to pot cultivations, the two invasive hosts can also be considered of at least moderate qual-

ity for *Rhinanthus alectorolophus* with an average biomass of individuals of about 220 mg and 150 mg on *Solidago* and *Symphyotrichum*, respectively. The biomass values per individual may be up to five times higher with the best host species in greenhouse pot cultivations (Těšitel et al. 2015b; Matthies 2021). However, the hemiparasitic plants in these cultivation experiments could benefit from optimal greenhouse conditions, including sufficient soil nutrients and reduced intraspecific competition due to the presence of only a single hemiparasite individual in each pot (Matthies 2021).

Both hemiparasitic species significantly suppressed the growth of both host species, which is the first experimental demonstration of an adverse effect of root hemiparasites on invasive species. We expected that the growth of the hemiparasites would correlate with the level of host suppression (hypothesis 2), which was only partially supported. Both hemiparasitic species reduced *Symphyotrichum* above-ground biomass by a third despite a significant difference in hemiparasite biomass (Figs 4, 5). Conversely, *Solidago* growth was reduced by 80% and 50% when parasitised by *Melampyrum* and *Rhinanthus*, respectively, corresponding to the difference in biomass production of the two hemiparasitic species and also to maximal levels of host biomass suppression reported from previous pot experiments (70% and 65% by *Melampyrum arvense* and *Rhinanthus alectorolophus*, respectively; Těšitel et al. (2015b); Matthies (2017); Sandner and Matthies (2018)). The difference in the host suppression could be related to their clonal growth characteristics, specifically the persistence of ramet connection. The clonal connections of *Solidago* ramets may persist for several years, while connections amongst *Symphyotrichum* ramets decay after one year (Schmid et al. 1995; Klimešová and Klimeš 2006). Schmid and Bazzaz (1987) suggested stronger physiological integration in *Solidago* due to the larger effects of experimental rhizome severance on *Solidago gigantea* growth than *Symphyotrichum*. The persistent clonal spread was identified as a significant positive predictor of hemiparasite-induced growth reduction (Demey et al. 2015); thus, the putatively stronger integration of *Solidago* ramets could be one of the reasons for the more extensive damage inflicted by the parasites. Physiological integration may be a trait contributing to a species' susceptibility to plant parasitism.

Examining the interactions between clonal hosts and hemiparasites presents a challenging task. Pot experiments are necessary to isolate the interaction between the host and the generalist hemiparasites from the natural community context, ensuring no other plant serves as a host. Typically, hosts are grown from seeds in these experiments, with hemiparasites later germinating in the pots or being transplanted as pre-germinated seedlings. Consequently, hemiparasites attach to young host individuals that have not yet developed clonal growth. Furthermore, arbitrary numbers of host and hemiparasite individuals (sometimes as low as one host with one hemiparasite) are used in most of the pot experiments (e.g. Cameron and Seel (2007); Rowntree et al. (2014); Těšitel et al. (2015b); Matthies (2017); Sandner and Matthies (2018); but see, for example, Matthies (1995) and Hejduk et al. (2020) for hemiparasite density-manipulation experiments). These issues limit the potential of such experiments to elucidate the clonal host–hemiparasite interaction because, in natural communities, hemiparasite seedlings mostly attach to mature individuals of perennial plants with a

fully-developed root system and clonal-growth organs. Thanks to transplanting whole soil blocks from the host population, our experiment maintains the host plant properties (developmental stage, ramet density) as close to natural conditions as possible. In addition, the hemiparasite seedlings were permitted to develop under natural climatic conditions, host phenological development and at densities close to realistic values (van Hulst et al. 1987; Mudrak and Lepš 2010). Hence, our experiment paves the way to more realistic pot experiments studying clonal host–hemiparasite interactions, which are of particular significance in European grassland ecosystem (Demey et al. 2015; Lepš and Těšitel 2015; Těšitel et al. 2017).

Conservation perspective

Pronounced biomass suppression of *Solidago* and *Symphyotrichum* is noteworthy from the restoration perspective. Both species are invasive, often achieving dominance and significantly impacting above-ground diversity (Hejda et al. 2021; Cubino et al. 2022; Fenesi et al. 2023). *Solidago* spp. also affected below-ground soil properties and the activity and biomass of soil bacteria and fungi (Zhang et al. 2009a, b; Scharfy et al. 2010; Pergl et al. 2023). Both species are listed in the second most serious category in the Black List of invasive species (BL2) with a massive environmental impact (Pergl et al. 2016). Hence, reducing their populations is crucial, particularly at sites of high conservation values. Mowing twice, a standard management technique for vegetation infested with *Solidago gigantea*, reduces the species' dominance. Cover reduction by 75% of the initial cover was reported over the long term, but the species is still persistent in the vegetation (Nagy et al. 2020; Szymura et al. 2022). A more pronounced suppression of *Solidago* may be achieved through cattle and sheep grazing or flooding (> 95% suppression; Nagy et al. (2020)). Despite the rapid spread of *Symphyotrichum lanceolatum* in wetland habitats of high natural value (Lanta et al. 2022), no information on managing this invasion is available. Biological control by introducing specialised insects or fungi from the species' native range has not been established yet in the invaded ranges, though several non-native insect enemies may be available in the case of *Solidago* (Fontes et al. 1994; Sheppard et al. 2006). Another biocontrol option available in subtropical regions may represent the widely-spread fungus *Sclerotium rolfsii*, causing the southern blight disease. Wilting of *Solidago canadensis*, induced by this fungus, has been reported from China (Tang et al. 2010) and the fungus application combined with soil disturbance led to 90% decrease in *Solidago canadensis* stem density (Zhang et al. 2019).

Using native hemiparasitic plants in combination with standard mowing management may offer another tool for the biocontrol of the two study species without any potential risks of introducing alien organisms to the ecosystems. The effects of hemiparasites on the invasive hosts observed in our experiment are comparable to the level of the suppression of *Calamagrostis epigejos* by *Rhinanthus alectorolophus* reported in previous research (Těšitel et al. 2017). The reduction and even elimination of this expansive grass by *Rhinanthus* have been established as a standard tool of biodiversity restoration in nature conservation in the Czech Republic (SPPK D02 002 2021). In

contrast, *Melampyrum arvense* has not been used in ecological restoration so far, possibly because it is now considered a vulnerable species confined mainly to steppes and protected areas in Central Europe (Těšitel et al. 2015a). However, this species used to be a noxious weed in winter cereal fields (e.g. Rau (1970); Çetinsoy (1980); Matthies (1995)). It can increase its biomass by 1/3 in nutrient-rich soil and prefers hosts from nutrient-rich environments (Matthies 2017). Such ecological characteristics align with the ecology of *Solidago* and *Symphyotrichum*, sometimes called ‘old-field perennials’ (Schmid and Bazzaz 1987; Schmid et al. 1995), which efficiently colonise bare ground, fallows and disturbed urban areas and thrive in humid, nutrient-rich soils. Our experimental results demonstrate the ability of both hemiparasites to suppress the invasive species, but implementing this finding in ecological restoration requires further testing in the field conditions over longer periods.

Genotype adaptation

We identified transgenerational effects in hemiparasitic interactions thanks to using hemiparasite seeds of the same population origin, but cultivated for two generations (= years) with the target host. The effects were not universal across all host–hemiparasite combinations; however, where present, they generally supported our hypothesis 3. Specifically, when the hemiparasites were exposed to the target host species during two previous generations, the offspring plants produced relatively more biomass (Fig. 6) or were more detrimental to the host (Fig. 7) in some host–hemiparasite combinations. The effects were more pronounced on the hemiparasite side of the association, a pattern identified in a previous study on genotype effects in root-hemiparasitic interactions (Rowntree et al. 2014). Two mechanisms may be at play here: classical genetics and the selection of alleles that provide better compatibility with a host species or epigenetic (maternal) effects acting in the same way (Anastasiadi et al. 2021). We are not able to distinguish between these two with the current data. Even in model organism studies, the state-of-the-art methodology struggles to provide absolute separation of selection and epigenetics (Schmid et al. 2018). However, any adaptive process facilitating the association with novel hosts is crucial for the biotic resistance role of the parasites.

The existence of transgenerational effects in host–hemiparasite compatibility suggests that the breeding of genotypes more compatible with the target invasive hosts or exposing the mother plants to the novel host species may potentially increase the success of biocontrol applications, at least in the case of *Rhinanthus*. The feasibility of such an approach is also supported by the observations of rapid adaptive evolution in *Rhinanthus alectorolophus* in response to environmental conditions and host species (Zopfi 1993; Pleines et al. 2013; Moncalvillo and Matthies 2023). The genetic diversity of hemiparasites was also demonstrated to be a significant predictor of their establishment success and fitness when cultivated with multiple host species (Rowntree and Craig 2019). Therefore, while breeding hemiparasites in monospecific host stands may be efficient for specific purposes, it is equally important to preserve the genetic diversity of the populations of hemiparasitic species in nature and in seed production

for ecological restoration; for instance, by cultivating hemiparasites with various host species from different plant functional groups so that the pool of genotypes efficient in various host–hemiparasites combinations is not depleted.

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Supplementary material I

Supplementary information

Authors: Tamara Těšitelová, Kateřina Knotková, Adam Knotek, Hana Cempírková, Jakub Těšitel

Data type: docx

Explanation note: **appendix S1:** Location of source localities of hemiparasite seeds and invasive host plants. **appendix S2:** Overview of the number of hemiparasite specimens and their biomass in the experimental treatments. **appendix S3:** Overview of host shoot counts and host biomass in the experimental treatments. **appendix S4:** Photographic documentation of all pots representing the experimental treatments.

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Supplementary material 2

Primary data table

Authors: Tamara Těšitelová, Kateřina Knotková, Adam Knotek, Hana Cempírková, Jakub Těšitel

Data type: xlsx

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Distribution and genetic diversity of the invasive pest *Halyomorpha halys* (Hemiptera, Pentatomidae) in Belgium

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Abstract

The brown marmorated stink bug, *Halyomorpha halys*, native to East Asia, is an invasive pest of economic importance. It has invaded North America and many European countries and is further expanding its range. In Belgium, it was first observed in 2011. *Halyomorpha halys* is known to cause severe damage in commercial fruit orchards and vegetable crops. A dramatic and unmitigated expansion of *H. halys* in its adventive range could lead to significant economic implications for agricultural production. In this study, occurrence data of *H. halys* since its first observation in Belgium was analysed together with molecular information to map the populations and evaluate the genetic diversity of this pest. The genetic diversity of *H. halys* in Belgium was compared to data from other invaded and native countries reported in previous studies to identify possible invasion routes. The analysis of 1176-bp of mitochondrial DNA cytochrome c oxidase I and II genes (COI and COII) led to the discovery of two novel COI-COII haplotype combinations currently unique to Belgium. The invasion of *H. halys* in Belgium is likely the result of multiple and ongoing introductions from its native region and from already invaded countries in Europe, particularly Italy. The expansion of the brown marmorated stink bug populations in Belgium is recent and ongoing. Presently, it appears to thrive best in northern Belgium.

* These authors contributed equally to this work.

Keywords

brown marmorated stink bug, haplotype diversity, Heteroptera, population genetics

Introduction

Halymorpha halys (Hemiptera: Pentatomidae) or the brown marmorated stink bug is a stink bug of economic importance, native to East Asia and an invasive pest species in Europe, North America, and other regions (Hoebeke and Carter 2003; Leskey et al. 2012; Lee et al. 2013; Haye et al. 2015; Zhu et al. 2016; Hamilton et al. 2018). In Europe, *H. halys* has already been detected as far north as The Netherlands (Aukema et al. 2019), Belgium (Claerebout et al. 2018) and the north of Germany (Hartung et al. 2022). Due to its polyphagous nature, *H. halys* can feed on virtually all primary fruit and vegetable crops in its invaded areas (Kuhar et al. 2012; Haye et al. 2015). For example, in northern Italy, it has become a key pest in fruit orchards, with an estimated economic impact of €588 million on the production of pear, apple, peach, and kiwi in 2019 (Bulgarini et al. 2020). *Halymorpha halys* causes damage through its feeding activity. It inserts its stylets into the plant and injects saliva, pre-digesting the plant tissue before feeding on it (Rice et al. 2014). This feeding behaviour causes scarring, deformities, pitting, and discolouration of the product, rendering it unmarketable or even inedible (Rice et al. 2014; Bulgarini et al. 2020). Since its first record in Belgium in 2011, observations of *H. halys* on public citizen science databases (Observation International and local partners 2022) only appeared in 2017. Currently, it is assumed that *H. halys* has established univoltine overwintering breeding populations in Belgium (Claerebout et al. 2018). An observation of *H. halys* from the Haspengouw region in July 2021 indicated that *H. halys* is already present in commercial pip fruit orchards in Belgium. However, to date, no dramatic population increases, mass occurrences or damage has been reported in fruit production in Belgium (Berteloot et al. 2021). In 2022, in Belgium, 219.000 t of apples and 366.000 t of pears, some of the most vulnerable fruits to *H. halys*, were produced (European Commission 2022a, b). Apples and pears represent a combined revenue of more than € 148 million of which pear production is the most significant part with a revenue of € 119 million (Verbond van Belgische Tuinbouwcoöperaties 2022). An unmitigated expansion of *H. halys* in Belgium could therefore have a considerable impact on fruit production, especially in the Flanders region, which produces most of the apples, pears, and soft fruits in the country.

The increased accessibility and affordability of molecular technologies, as well as the expansion of databases containing publicly available DNA sequence data, have aided in the use of molecular tools to assess the genetic diversity and potential origin of invasive species (Cristescu 2016; Hamelin and Roe 2019). Due to its lack of recombination, relative neutrality and shorter coalescence time, mitochondrial DNA (mtDNA) is a highly popular molecular marker for examining genetic diversity and phylogeography of (invasive) animal species (Ficetola et al. 2008; Rollins et al. 2011;

Bras et al. 2019; Ryan et al. 2019). Furthermore, for invasive species, the accumulation of easily reproducible genetic data in public databases facilitates the regular addition of new data from the invasion front to build a global pattern of invasion dynamics. For example, for *H. halys*, mtDNA sequences (portions of the mitochondrial gene cytochrome c oxidase subunits I and II – COI and COII, respectively) are already available for both native (China, Japan, Korea) and invaded countries (Garipey et al. 2014; Xu et al. 2014; Cesari et al. 2015; Cesari et al. 2018; Lee et al. 2018; Yan et al. 2021).

In this study, we set out to fill one of the geographical knowledge gaps in Europe on the genetic diversity and distribution of *H. halys*. First, we investigated the genetic diversity of *H. halys* in Belgium by sequencing 99 specimens from 18 locations at two mitochondrial genes: COI and COII. Then, we compared those sequences to previously published *H. halys* sequences to infer the putative invasion routes to Belgium. Lastly, we used public citizen occurrence data to map the distribution of *H. halys* in Belgium from 2020 to 2022 and gain demographic insights into the Belgian populations.

Methods

Insect sampling

Halyomorpha halys specimens were collected from 18 locations in Belgium (Suppl. material 1) from agricultural sites and private gardens in 2021 and 2022 using baited traps (dual lure from Trécé Inc., Adair Oklahoma, USA) or by beating of the lower part of a tree trunk/shrub. The collected samples were stored dry or in 70% ethanol at -20 °C until used for molecular analysis.

DNA extraction & sequencing

DNA was extracted from two legs using a Chelex extraction method (Walsh et al. 1991). Briefly, the legs were crushed with a sterile pestle in 100 µl of 5% Chelex 100 (Bio-Rad, USA) solution. They were then incubated at 85 °C for 90 min, and the supernatant was collected after 3 min of centrifugation at 12,000 rpm. The cytochrome oxidase subunit I (COI) and II (COII) barcode regions of the mitochondrial DNA were amplified through PCR. For the COI region, the LCO 1490 (5'- GCT-CAACAAATCATAAAGATATTTGG-3') and HCO 2198 (5'- TAAACTTCAGGGT-GACCAAAAAATCA-3') primers (Folmer et al. 1994) were used. For COII, the HhalsCO2F2 (5'-TAACCCAAGATGCAAATTCT-3') and HhalsCO2R2 (5'- CCATA-TATAATTCCTGGACGA-3') primers (Xu et al. 2014) were used. For both regions, the following PCR cycles were used: initial denaturation at 94 °C for 3 min, 38 cycles of denaturation at 94 °C for 30 sec, annealing at 48 °C for 30 sec and extension at 72 °C for 45 sec, followed by a final extension step at 72 °C for 7 min. The PCR product quality was checked on agarose gel. Both strands (forward and reverse) for each barcode region were sequenced (Eurofins, Germany GmbH) by Sanger sequencing.

Haplotype diversity analysis

Forward and reverse sequences were trimmed and assembled into a consensus sequence using CodonCode Aligner (version 10.0.2). COI and COII sequences obtained in this study were compared to COI and COII sequences from Yan et al. (2021) and Cesari et al. (2018), retrieved from GenBank and BOLD. Other previously conducted studies on the genetic diversity of *H. halys* only sequenced COI or COII fragments (or only reported unique haplotypes found without specifying which samples are associated with which haplotypes) (Garipey et al. 2014; Xu et al. 2014; Valentin et al. 2017; Lee et al. 2018; Kapantaidaki et al. 2019). All individual COI and COII haplotypes found in this study have already been reported in other publications (see results) and were given the haplotype name used in those publications (Cesari et al. 2018; Yan et al. 2021). However, for COI, there were discrepancies between the sequences of haplotypes with the same name found in Cesari et al. (2018) and Yan et al. (2021). Haplotypes found in our study were named after the reference haplotypes of the study of Valentin et al. (2017) if a sequence match was found. Haplotype names from Cesari et al. (2018) were distinguished with the letter “c” (Table 1). The sequence of haplotype H41 from Yan et al. (2021) perfectly matched the H41 haplotype from Valentin et al. (2017) but corresponded to the sequence of H46 from Cesari et al. (2018). Therefore, the name of the sequence of this haplotype was retained in the present study as H41. Another sequence found in this study without a match in Valentin et al. (2017) or Yan et al. (2021) was already named H41 in Cesari et al. (2018) and was renamed “H41c” to distinguish it. For H40 and H43, the same haplotype names were given to different sequences by Cesari et al. (2018) and Yan et al. (2021). Again, those sequences were compared to the ones of Valentin et al. (2017). The H40 and H43 sequences from Yan et al. (2021) perfectly matched the identical name sequences from Valentin et al. (2017), and these names were retained in the present study. A letter “c” was added to the H40 and H43 sequences from Cesari et al. (2018) to distinguish them. Finally, the H42 and H49 sequences from Cesari et al. (2018) did not match the H42 and H49 haplotypes from Valentin et al. (2017) (no H42 and H49 haplotypes in Yan et al. (2021) for comparison). To be consistent, the letter “c” was added to the H42 and H49 sequences from Cesari et al. (2018).

Table 1. Summary of the discrepancies between sequences of COI-haplotypes with the same name in different studies and the names used in the present study.

Valentin et al. (2017)	Cesari et al. (2018)	Yan et al. (2021)	This study
H40	/	H40	H40
/	H40	/	H40c
H41	H46	H41	H41
/	H41	/	H41c
/	H42	/	H42c
H43	/	H43	H43
/	H43	/	H43c
/	H49	/	H49c

Sequences were aligned and analysed in R v4.0.2 (R Core Team 2020), using packages *msa* (Bodenhofer et al. 2015), *adegenet* (Jombart 2008) and *pegas* (Paradis 2010). The relationships among haplotypes within Belgium and between haplotypes from Belgium and other countries were investigated through haplotype networks and genetic diversity indexes (haplotype, Hd, and nucleotide diversity, π). We tested for the presence of a genetic structure in Belgium by performing a Mantel test (Euclidean) on genetic and geographic distance matrices between collected specimens.

Occurrence data

Occurrences from January 1st, 2017, to December 31st, 2022 were obtained from the publicly available citizen science database (Observation International and local partners 2022). For *H. halys*, the recorded occurrences on the public citizen science database are quality-controlled, and the taxonomic accuracy of each observation is confirmed or rejected by a specialist through photos. The occurrence data was additionally examined to check the validity of all the occurrences. Only occurrences with a high confidence level in the taxonomic identification of *H. halys* and with spatial coordinates were used. Additionally, specimens collected during this research were identified through morphological identification using the *H. halys* identification key from Maistrello et al. (2016) and confirmed by our molecular analysis. The occurrence data was visualised with QGIS 3.30.1 (QGIS Development Team 2023).

Results

DNA sequences

- COI: 99 *H. halys* sequences were retrieved out of 99 specimens collected. All specimens yielded a 658-bp DNA sequence. The DNA sequence data and specimen collection information were made accessible on GenBank with accession numbers OR581617–OR581715.
- COII: 93 sequences with a length of 518-bp were retrieved from 99 *H. halys* specimens collected. The DNA sequence data and specimen collection information were made accessible on GenBank with accession numbers OR602454–OR602546.

Haplotype diversity of *H. halys*

For the COI fragment individually, nine distinct haplotypes were found, consisting of 14 polymorphic sites (Fig. 1a). Three haplotypes were dominant (H01, H03 and H08) and were shared by roughly 80% of the individuals collected. Haplotype diversity was 0.77 ± 0.02 (mean \pm SD). The obtained COI sequences from this study were compared with 609 COI sequences from other European countries, Chile, the USA, China, and Japan. H01 and H03 haplotypes are shared with those in invaded areas in

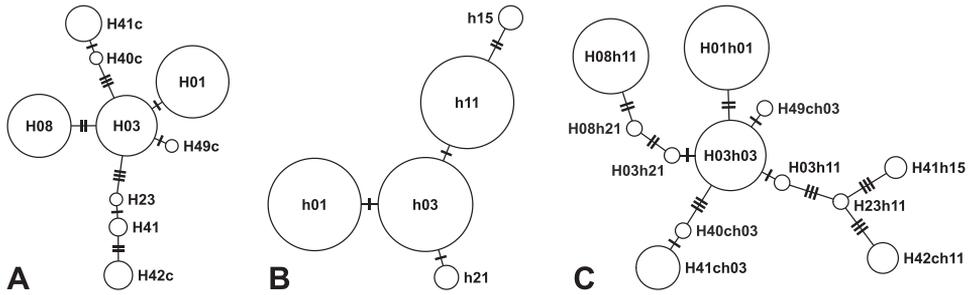


Figure 1. Haplotype network using **A** COI **B** COII and **C** COI-COII fragments of *H. halys* in Belgium. The circle size is proportional to the square root of the frequency of the haplotypes. The tick marks on each line represent a base pair difference.

Europe and America, as well as with those in the native area of China, but not with Japan. Haplotype H08 is shared only with some invaded areas in Europe (Austria and Italy). The remaining haplotypes are shared with Italy (H40c, H41, H41c, H42c and H49c), Japan (H23 and H41) and Hungary (H41).

For the COII fragment individually, five distinct haplotypes were found, consisting of 5 polymorphic sites (Fig. 1b). Haplotype diversity was 0.70 ± 0.01 . Our COII sequences were compared with 651 COII sequences from other European countries, Chile, the USA, China, and Japan. Haplotypes h01, h03 and h11 accounted for 91 out of 95 haplotypes from Belgium. Haplotypes h01 and h03 are shared with invaded areas in Europe, the USA and China but not with Japan. Haplotypes h11 and h15 are only shared with some other European countries (Austria, Hungary, and Italy) and Japan. Haplotype h21 is shared only with Italy.

The resulting concatenated 1176-bp sequences rendered 12 distinct COI-COII haplotypes among 95 specimens consisting of 22 polymorphic sites in total (Fig. 1c). Two novel COI-COII haplotypes were identified (H03h11 and H08h21) in our samples; these haplotypes are unique to Belgium. The ten other haplotype combinations were previously reported in the literature. The comparison of COI-COII haplotypes from Belgium to previously published sequences confirm the pattern observed for individual loci. The most frequent haplotype H01h01 is shared with all native and invaded countries included in this study (Table 2). H03h03, the second most frequent haplotype, has been recorded in Austria, Chile, China, Italy, and Hungary. Some of the remaining haplotypes (H03h11, H03h21, H41ch03 and H49ch03) are only shared with Italy (and Austria for H08h11) and are close to haplotypes from China. Others (H23h11, H41h15 and H41ch11) are shared with Italy (and Hungary for H41h15) and/or with (or close to) Japan (H46h15, H42h11, H23h11 and H08h11). Finally, the H08h21 and the H03h11 haplotypes are unique to Belgium and are close to the haplotypes from Italy (within the group of haplotypes found in most invaded countries and China).

Table 2. Summary table of mtDNA (COI-COII) diversity by country. With N: sample size, Hn: number of haplotypes, h: haplotype diversity and π : nucleotide diversity (only countries with available COI-COII sequences are listed).

Continent	Country	First record	N	Hn	Hd \pm SD	$\pi \pm$ SD	Study
Asia	China	Native	90	24	0.86 \pm 0.02	0.0033 \pm 0.0018	Yan et al. 2021
	Japan	Native	65	32	0.94 \pm 0.01	0.0024 \pm 0.0014	Yan et al. 2021
	Turkey	2017 (Güncan and Gümüş 2019)	11	1	0	0	Yan et al. 2021
Europe	Austria	2015 (Rabitsch and Friebe 2015)	15	4	0.69 \pm 0.10	0.0021 \pm 0.0013	Yan et al. 2021
	Belgium	2011 (Claerebout et al. 2018)	95	12	0.79 \pm 0.02	0.0031 \pm 0.0018	This study
	Georgia	2015 (Gapon 2016)	31	1	0	0	Yan et al. 2021
	Greece	2011 (Milonas and Partinevelos 2014)	8	3	0.61 \pm 0.16	0.0025 \pm 0.0017	Cesari et al. 2018
	Hungary	2014 (Vétek et al. 2014)	90	3	0.11 \pm 0.04	0.0003 \pm 0.0003	Yan et al. 2021
	Italy	2012 (Maistrello et al. 2014)	16	18	0.72 \pm 0.03	0.0028 \pm 0.0016	Cesari et al. 2018; Yan et al. 2021
	Romania	2015 (Macavei et al. 2015)	30	1	0	0	Cesari et al. 2018
	Serbia	2015 (Šeat 2015)	13	5	0.61 \pm 0.07	0.0014 \pm 0.0008	Yan et al. 2021
	Slovenia	2017 (Rot et al. 2018)	15	3	0.51 \pm 0.12	0.0012 \pm 0.0008	Yan et al. 2021
North America	United States	2001 (Hoebeke and Carter 2003)	24	1	0	0	Yan et al. 2021
South America	Chile	2017 (Faúndez and Rider 2017)	31	2	0.06 \pm 0.06	0.0001 \pm 0.0002	Yan et al. 2021

The results of our Mantel test indicated a significant but weak correlation ($r = 0.14$, $p = 0.001$) between the genetic distance and the geographical distances for pairs of individuals. High haplotype diversity was observed in Belgium, $Hd = 0.79 \pm 0.02$, with a nucleotide diversity value of $\pi = 0.0031 \pm 0.0018$ (Table 2). This level of genetic diversity was lower than that of native regions Japan and China ($Hd = 0.94 \pm 0.014$ and 0.86 ± 0.023 , respectively) but similar to Italy ($Hd = 0.72 \pm 0.033$) (Table 2). Other invaded areas of *H. halys* typically had lower haplotype diversity but their estimation may be approximate due to shallower sampling depth (low sample size and/or few sampling locations; Table 2). The genetic data analysis of this research is publicly available through the following link: <https://zenodo.org/records/10210286>.

Distribution of *H. halys* in Belgium

The 740 observations from 6 years (2017–2022) were checked for accuracy and completeness. Since the first record in 2011, occurrences in subsequent years initially remained low, without any public citizen database records or specimens collected until 2017. *Halyomorpha halys* was recorded once in 2017 and 2018, 5 times in 2019 and 35 times in 2020. In recent years, the number of observations of *H. halys* has increased dramatically. In 2021, 183 observations were recorded, followed by a substantial increase to 515 in 2022. Up until November 2023, the Belgian public citizen database reported more than 2200 observations, a more than tenfold increase compared to 2021. From the occurrences of *H. halys* in 2020–2022, most of the observations were made in northern Belgium, mainly around the urban areas of the cities of Gent, Leuven, and Mechelen and the region of Haspengouw (Fig. 2a–c).

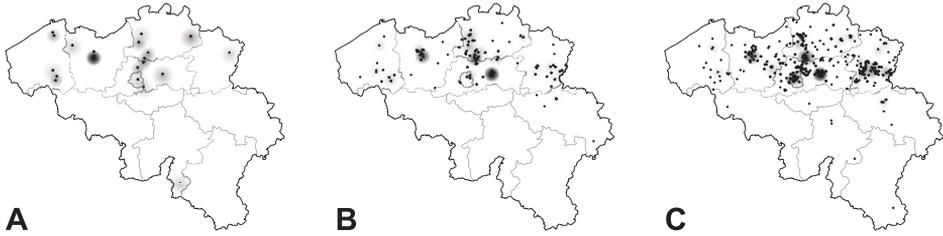


Figure 2. Map of Belgium with recorded occurrences of *H. halys* in **A** 2020 **B** 2021, and **C** 2022. Each dot is an individual record, and density clouds indicate the level of density of occurrences in one area.

Discussion

This study uncovered 9 COI, 5 COII and 12 COI-COII distinct haplotypes from 99 *H. halys* specimens collected in Belgium. Among these, two new COI-COII haplotype combinations (H03h11 and H08h21), currently unique to Belgium, were observed.

The principal COI haplotypes present in Belgium were H01 (33%), H08 (24%) and H03 (23%). H01 and H03 are the most frequent haplotypes in China and in most invaded countries (Garipey et al. 2014; Valentin et al. 2017; Cesari et al. 2018; Lee et al. 2018; Yan et al. 2021; Garipey et al. 2021). As such, their presence in Belgium is to be expected and therefore does not help to determine the origin of *H. halys* in Belgium. Interestingly, however, H08 is the second most abundant haplotype in Belgium. So far, this haplotype has been exclusively detected in certain European countries: Austria, France, Italy, Switzerland and Serbia (Valentin et al. 2017; Cesari et al. 2018; Garipey et al. 2021; Yan et al. 2021). This suggests that at least part of the *H. halys* population in Belgium originates from previously invaded territories in Europe (i.e., bridgehead effect; Lombaert et al. 2010). Remarkably, eight out of the nine COI haplotypes found in Belgium are shared with Italy. Furthermore, half of these haplotypes were reported exclusively in these two countries (H40c, H41c, H42c and H49c) suggesting that Italy is a direct region of origin of *H. halys* in Belgium. The rare H23 haplotype was only found once in Belgium and has been reported in Japan and the Western USA (Oregon) (Valentin et al. 2017). It was also detected in a shipment in Australia originating from Italy, suggesting that the H23 haplotype is also present in Italy (Horwood et al. 2019; Garipey et al. 2021). Introductions may thus have originated from Japan or the USA in addition to Italy.

For the COII fragment, h01, h03 and h11 each accounted for 32% of the abundance. The h01 and h03 haplotypes are native to China and Korea (but have not been reported in Japan yet) and are the most frequent COII haplotypes in invaded countries (Xu et al. 2014; Cesari et al. 2018; Yan et al. 2021). The distribution of the h11 COII haplotype is more restricted and has only been reported in Austria, Italy, Japan, and Korea (Xu et al. 2014; Cesari et al. 2018; Yan et al. 2021). Consistent with Italy as a source of *H. halys* for Belgium, the h21 haplotype was isolated from two specimens collected in Belgium and is only shared with Italy (Cesari et al. 2018).

In this study, the combination of COI and COII fragments did not result in a significantly better geographic resolution to reveal possible origins of the invasion.

However, the presence of haplotype H03h21 provides some additional support for Italy as a source of the invasion since this combination is only shared with a sample from the Piedmont region of Italy (Cesari et al. 2018). Additionally, the inclusion of the COII fragment in this study revealed two novel haplotype combinations unique to Belgium: H08h21 and H03h11. The former is likely to originate from Italy since haplotype h21 has been exclusively reported there (Piedmont region, same sample as for H03h21), while H08 has been detected multiple times (Piedmont and Lombardy regions of Italy). H03h11 is more singular because it combines a COI haplotype native to China (H03) and a COII haplotype native to Korea or Japan (h11; see above). Such a combination could arise from recent COI or COII mutations. H03 is only two substitutions from the closest COI haplotype native to Japan (H27), and h11 is only one substitution from three COII haplotypes native to China (h03, h05 and h10). Alternatively, this combination could also exist in the regions of origin (China and/or Japan/Korea) but has not yet been sampled. Belgium's dominant haplotypes generally overlap with those from previously invaded European countries, such as Italy, Switzerland, Austria, and Hungary. In contrast, rarer haplotypes are exclusively shared with Italy and its native region of Japan (H23).

The genetic diversity found in Belgium is surprisingly high ($H_d = 0.79$, $\pi = 0.0018$, $N = 95$) compared to other invaded countries previously studied (Table 2), several distinct haplotypes were isolated from single sampling sites (Suppl. material 1). A reduced genetic diversity is usually expected for introduced species because of a limited number of founders associated with early genetic drift. However, genetic bottlenecks associated with introductions can be counteracted if many individuals are introduced at the same time or if repeated introductions occur (Dlugosch and Parker 2008). Similarly, Schuler et al. (2020) found a high haplotype diversity ($H_d = 0.68$, $\pi = 0.0046$, $N = 156$) in the *H. halys* population of South Tyrol in Northern Italy. The population in Tyrol was established by a secondary introduction from Eastern USA into the Emilia Romagna region of Italy and the spread of the founder population in Switzerland to the neighbouring countries. In contrast, Valentin et al. (2017) concluded that most invasive populations in North America and Europe were established from a direct introduction of *H. halys* from China with separate introductions into Eastern and Western USA and Canada, as well as into Switzerland and Greece. The high genetic diversity of *H. halys* populations in Belgium and the overlap of haplotypes with previously invaded European countries, such as Italy, Switzerland, Austria, and Hungary but also with Japan indicates that the Belgian populations probably originated from multiple invasions from already invaded European countries, mainly Italy, but also directly from Japan through inadvertent human-mediated transportation (often due to global trade of goods) (Valentin et al. 2017). The weak genetic structure detected by our Mantel test could be related to the recent local expansion of these genetically diverse clusters.

Previous studies have shown that citizen science provides valuable data to characterise the spread of *H. halys* (Maistrello et al. 2016; Stoeckli et al. 2020; Streito et al. 2021). From our data in Belgium, more *H. halys* observations are recorded in the northern part of Belgium compared to the southern part of Belgium, with more urban

areas than agricultural, forested, and rural areas. However, on a national scale, spatial biases are commonly associated with human population density, settlements, and road infrastructure (Kelling et al. 2015; Geldmann et al. 2016; Girardello et al. 2019). The bias towards urban areas in citizen-collected occurrence data and rising popularity of tools to make observations (e.g., smartphones) represents a challenge to infer true spatio-temporal patterns from our occurrence data (Bowler et al. 2022). Nevertheless, it also provides natural experimental gradients to examine the impacts of future environmental scenarios including climate change (Lahr et al. 2018). Urban areas are often seen as heat islands, offering more suitable refugia to survive winters or more favourable conditions throughout the seasons to develop and reproduce. Therefore, these urbanised areas often comprise a larger population of insects (Kaiser et al. 2016; Frank and Backe 2023). According to Kistner (2017), the northern part of Belgium lies near the latitudinal border of the climate in Europe suitable for the winter survival and summer reproduction of *H. halys*. In contrast, the southern part of Belgium is currently modelled to be an unsuitable eco-climatic region for *H. halys*, with colder summer and winter temperatures and fewer refugia to survive winter due to the lower urbanisation (Kistner 2017). Niche modelling through climate suitability by Streito et al. (2021) essentially confirms this, with the northern part of Belgium having more suitable areas for *H. halys* while the southern part is more marginal to unsuitable for its survival and expansion. Despite the possibility of spatio-temporal bias in our occurrence data, some temporal trends can still be derived from citizen-collected occurrence data (Powney et al. 2019; Outhwaite et al. 2020; Sheard et al. 2021; Zattara and Aizen 2021; Bowler et al. 2022). Our observational data shows an almost fifteenfold increase in observations from 2020 to 2022. However, to date, no damage related to *H. halys* has been reported in agricultural production in Belgium (Berteloot et al. 2021). Based on the occurrence data, it is likely that the population expansion of *H. halys* in Belgium is very recent, as a relatively high number of observations were only made in 2021, 2022 and 2023. Streito et al. (2021) define three main phases in the invasion of *H. halys*: (1) the very beginning of the invasion, when populations of *H. halys* are low, and naturalists and official monitoring services who are excellent observers and expecting the emergence of *H. halys* can detect its presence, (2) when abundance increases, non-naturalist citizens are able to detect the species and start to provide information on the dynamics of the invasion and indirectly on the level of populations through citizen science platforms, and (3) when populations have expanded and become large enough, agricultural professionals take over and can predict and assess the population density and damages. From our occurrence data, the *H. halys* population in Belgium is seemingly under expansion, being numerously recorded in the citizen science databases.

Lastly, the univoltine *H. halys* population in Belgium likely expands in the summer and declines in the winter in current climatic conditions due to Belgium being situated at the northern latitudinal border of climatic suitability for *H. halys*, with winters cold enough to kill more individuals than winter temperatures in southern European countries like Italy, possibly delaying the fast expansion of its populations. However, both parts of Belgium are modelled to be suitable for the survival and development of *H. halys* by 2100 (Kistner 2017).

Conclusions

The results of this study provide haplotype information for *H. halys* from a newly invaded region. The haplotype diversity in Belgium is surprisingly high, with 9 COI, 5 COII and 12 COI-COII haplotypes found. The invasion of *H. halys* in Belgium likely occurred repeatedly and is assumed to be still ongoing through human-mediated transportation from other invaded European countries and directly from its native regions in Eastern Asia. A significant overlap between Belgian and Italian haplotypes points to Italy as the most probable source for a significant proportion of haplotypes currently present in Belgium. By combining the citizen-collected occurrence data with the molecular data, we assume the population expansion of *H. halys* is recent and ongoing.

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Supplementary material I

Metadata of all samples

Authors: Olivier Hendrik Berteloot, Alexandre Kuhn

Data type: csv

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Field studies of the ecological impacts of invasive plants in Europe

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Abstract

The impacts of invasive species can vary widely across invaded sites and depend on the ecological variable of study. In this paper, we describe the first harmonised database that compiles scientific evidence of the ecological impacts of invasive plant species at continental scale. We summarise results from 266 publications reporting 4259 field studies on 104 invasive species in 29 European countries. For each study, we recorded whether the effects were statistically significant and noted their direction (i.e. decrease or increase in the response variable when compared to uninvaded sites). We classified studies, based on the impacts on the levels of ecological organisation (species, communities and ecosystems), taxa and trophic level. More than half of the studies were conducted in temperate and boreal forests and woodlands and temperate grasslands. Notably, one third of the studies focused on just five invasive species. Most studies were on native species followed by studies on communities. Impacts on plants were more frequently studied than impacts on other taxa and trophic groups. Overall, 43% of the studies reported significant impacts, with more significant decreases (26%) than increases (17%) in the response variables. Significant impacts were more frequent on species and communities than on ecosystems; and on plants than on animals or microbes. This database is of interest for academic, management and policy-related purposes.

Keywords

Biological invasions, context-dependence, diversity, ecological organisation, ecosystem properties, European Regulation on IAS, non-native plants, trophic level

Introduction

Many non-native species introduced by human agency outside their original area of distribution invade natural areas and cause ecological impacts to native species, communities and ecosystems (Simberloff et al. 2013). Ecological impacts are defined in this paper as any statistically significant ecological change occurring when an invasive species is present compared to when the invasive species is absent. This change can be a decrease (i.e. negative impact) or an increase (i.e. positive impact) of any ecological attribute of the invaded ecosystem (Jeschke et al. 2014). Thus, it is important to note that negative and positive impacts are independent of ethical and societal human values (Vimercati et al. 2020).

Information on the impacts of invasive species is of fundamental importance to assist management and policy (Vilà et al. 2019). In particular, empirical studies of ecological impacts provide essential scientific evidence to underpin risk assessment of invasions that are often used to rank and prioritise management actions. Despite the fast increase in the number of field studies testing for invasive species impacts, the majority focus on a few species and regions. Consequently, there are still important biases and gaps in knowledge that preclude our capacity to provide information for management and policy actions (IPBES 2023). It is thus of paramount importance to synthesise the scientific evidence on impacts to identify which are the most studied invasive species, the most studied habitats and the most studied impact types.

Meta-analyses have shown a strong context-dependency not only in the magnitude, but also in the direction of the impacts (Pyšek et al. 2012; Gallardo et al. 2016; Volery et al. 2021; Romero-Blanco et al. 2023). For example, an invasive N-fixing plant may strongly increase soil fertility in a recipient ecosystem with N-deficient soils and lacking native N-fixing species, but may have negligible impacts in communities including native N-fixing plants or in soils otherwise rich in N (Vitousek and Walker 1989; Castro-Díez et al. 2014, 2016). Moreover, invasive species can cause multiple, sometimes contrasting, impacts at different levels of ecological organisation (species, communities and ecosystems). For example, an invasive N-fixing plant may increase N soil availability and this can favour the establishment of some native plant species at the expense of others, with a neutral effect on species richness (Marchante et al. 2011). Thus, the impact of an invasive species can vary, presenting a neutral, negative or positive effect. This variability depends on factors such as the identity of the native species under study or whether the focus is on particular native species or the entire community. Therefore, to guide management decisions and biodiversity conservation efforts, it is important to document a broad spectrum of numerical increases and decreases in ecological responses following invasion. Given the conservation interest in native species and communities, adopting a value-laden perspective, their decrease may be considered deleterious, while an increase may be considered beneficial (Vimercati et al. 2020).

The environmental assessment of the impacts of invasive species requires the analysis of the full range of ecological changes after invasion. To this end, we conducted a comprehensive review of field studies reporting ecological impacts of invasive plant species in Europe to identify the most studied species, countries and habitats and to describe the frequency and direction of impacts. We classified impacts according to four

criteria: i) the ecological level at which the impact is measured, i.e. species, communities and ecosystems; ii) the affected taxonomic level, i.e. microbes, plants and animals; and iii) the trophic level of the affected taxa. Beyond identifying the most-studied invasive plant species and habitats and their most-studied impacts, our database also enables the exploration of differences in the frequencies and directions of impact types studied. Specifically, we explored if there were differences in the frequency of impacts amongst levels of organisation and taxa.

Material and methods

We started from the studies conducted in Europe extracted from the databases constructed and analysed in Pyšek et al. (2012) and in Castro-Díez et al. (2019). Additionally, we searched for new publications on the Web of Science (<https://www.webofscience.com/wos/alldb/basic-search>) database on 31 December 2022 with no restriction on publication year, using the following search term combinations: (plant inva* OR exotic plant OR alien plant OR non-native plant) AND (impact* OR effect*) AND (community structure* OR diversity* OR ecosystem process* OR competition*). Amongst the retrieved documents, we first screened titles and abstracts to identify all publications on the impacts of invasive plants conducted in Europe. We then examined each publication and constructed a database of impacts according to the following selection criteria:

1. The studies had to be in natural or semi-natural field conditions in Europe. The habitat type of the study was classified according to the IPBES unit of analysis (IPBES 2018) with the exception that, in this study, coastal areas was considered for terrestrial plants. The evidence of impact was based on observational or experimental (i.e. removal or addition of target species) field studies comparing simultaneously invaded or uninvaded sites where the identity of single invasive species of study was explicitly mentioned. We excluded tree plantations. We also excluded those referring to impacts by several invasive species.
2. When the same publication examined different response variables, different invasive species, different ecosystem types or geographically different localities, we considered each as different entries in the database (i.e. study, hereafter).
3. When a response variable was measured at different times (e.g. sampling species diversity across years), we made an informed decision on whether to take the mean value across times or to consider each measure as independent. However, when the variable was repeated across short periods (e.g. sampling N availability in different weeks), we only used the final measurement or the most representative (e.g. when the soil activity was the highest).
4. When the study manipulated other environmental factors in addition to invasion, we only considered results from the non-manipulated plots.
5. When the study investigated the effects of different degrees of invasion and different residence times (i.e. old vs. recent invasions), we examined differences between the least invaded sites and the most invaded sites and differences between uninvaded sites and sites with the longest time since invasion.

As all studies dealt with established non-native plant species and their threats to biological diversity and/or ecosystems, for simplicity, we refer to them as “invasive species” through out the text.

Following Vilà et al. (2011), impacts were classified according to the affected level of ecological organisation as follows: impacts to native species (e.g. abundance, performance, biomass), to communities (e.g. abundance, biomass or diversity) and to ecosystem properties (e.g. soil C/N, nutrient fluxes, decomposition rates, pH, nutrient pools, resource availability, soil minerals, soil organic matter and soil salinity/cation exchange capacity) (Table 1, Suppl. material 1: table S1). Furthermore, when the information was available, the impacts to species and communities were also classified according to the affected taxa (i.e. microbes, plants and animals) and to the trophic level of the affected taxa (i.e. impacts to herbivores, parasites, plants, pollinators, predators, omnivores, decomposers and symbionts) (Table 2). If the native species of concern belongs to different trophic levels along its life history, we considered the one during the stage of the study. In total, the database considered 23 impact types, which integrate the main biodiversity and ecosystem changes after invasion and allow for comparing impacts across studies (see Tables 1, 2).

For each impact, we recorded the statistical significance (no/yes) and direction (increase/decrease) of differences between invaded and uninvaded plots. For the purpose of this analysis, the direction does not mean a desirable/undesirable impact, but a significant increase or decrease of the response variable analysed in the invaded compared to the uninvaded control treatment, respectively.

To search for differences in the frequency of significant impacts across different levels of organisation (species, community, ecosystem) and taxa (animals, microbes, plants), we summed the number of responses – whether significant or non-significant – for each impact type. Responses were grouped, based on the identity of the invasive species and the respective publication.

We employed generalised linear mixed models (GLMMs) with a binomial (logit link function) error distribution family (lmerTest package; Kuznetsova et al. (2017)). The response variable was a two-column matrix generated using the ‘cbind’ function to combine the counts of significant and non-significant impacts. In each model, we included as a fixed factor the levels of ecological organisation or taxa. To account for the non-independence of data, we included the publication and the identity of the invader as random factors. Post-hoc Tukey tests (emmeans package; Russell (2018)) were then applied to evaluate differences in impact frequencies amongst levels of ecological organisation and taxa. To visualise these differences, we used the package ggeffects (Lüdtke 2018). All analyses were performed in R (v. 4.2.1, R Core Team (2022)).

Open research statement

All data employed in this research are archived in Figshare repository <https://doi.org/10.6084/m9.figshare.23579082>.

Table 1. Ecological impacts of invasive plant species studied in field conditions in Europe classified by categories of ecological organization (species, communities, ecosystems), with indication of the response variables examined in the literature. In parenthesis, the sample size (number of field studies testing for impacts). See Table S1 for definitions of the impact type classification.

Level of ecological organization	Impact type	Variables related to
Species (576)	Animal (176)	Animal abundance (143), activity (10), fitness (4), performance (19)
	Microbial (5)	Microbial abundance (5)
	Plant (395)	Plant abundance (223), biomass (34), fitness (66), performance (72)
Community (2541)	Animal (1142)	Animal abundance (682), activity (3), biomass (11), diversity (446)
	Microbial (370)	Microbial abundance (111), activity (150), biomass (17), diversity (92)
	Plant (1016)	Plant abundance (254), biomass (130), diversity (632)
Ecosystem (1155)	Soil carbon to nitrogen ratio (C/N) (74)	C/N (74)
	Nutrient fluxes (25)	C fluxes (11), N fluxes (14)
	Decomposition rates (39)	Litter decomposition (38), soil organic matter mineralization (1)
	pH (134)	pH (134)
	Nutrient pools (402)	C pools (114), N pools (194), P pools (94)
	Resource availability (83)	Light (19), moisture (60), soil temperature (4)
	Soil minerals (264)	Soil minerals (264)
	Soil organic matter (85)	Soil organic matter (85)
Soil salinity/cation exchange capacity (CEC) (49)	Soil CEC (1), salinity (3), salinity/CEC (45)	

Table 2. Ecological impacts of invasive plant species studied in field conditions in Europe classified by the trophic level of affected species (i.e. decomposers, herbivores, omnivores, parasites, plants, pollinators, predators, symbionts) with indication of the response variables examined in the literature. In parenthesis, sample size (number of field studies testing for impacts).

Trophic level of the affected taxa	Variables related to
Decomposer (269)	Decomposer abundance (189), biomass (7), diversity (62), activity (11)
Herbivore (100)	Herbivore abundance (62), diversity (36), performance (2)
Omnivore (47)	Omnivore abundance (41), diversity (3), fitness (2), performance (1)
Parasite (50)	Parasite abundance (44), biomass (2), diversity (4)
Plant (1411)	Plant abundance (477), biomass (164), diversity (632), fitness (66), performance (72)
Pollinator (353)	Pollinator abundance (190), activity (9), diversity (142), fitness (2), performance (10)
Predator (287)	Predator abundance (224), activity (4), biomass (1), diversity (54), performance (4)
Symbiont (23)	Symbiont abundance (16), biomass (1), diversity (6)

Results

Our final database included 266 publications describing 4259 field studies of 104 invasive plant species in Europe (Fig. 1, Suppl. material 1: tables S2, S3).

Impacts of invasive plants are widely studied across Europe, although around 50% of studies were carried out in six countries (Spain, Poland, Czech Republic, Germany, Italy and Portugal) and there are some countries without any studies (e.g. Albania, Bulgaria, Estonia and Latvia) (see Fig. 2). Most studies were conducted in temperate and boreal forests and woodlands (33%) and temperate grasslands (26%), followed by coastal areas (14%) and Mediterranean forests and woodlands (12%) (Fig. 3).

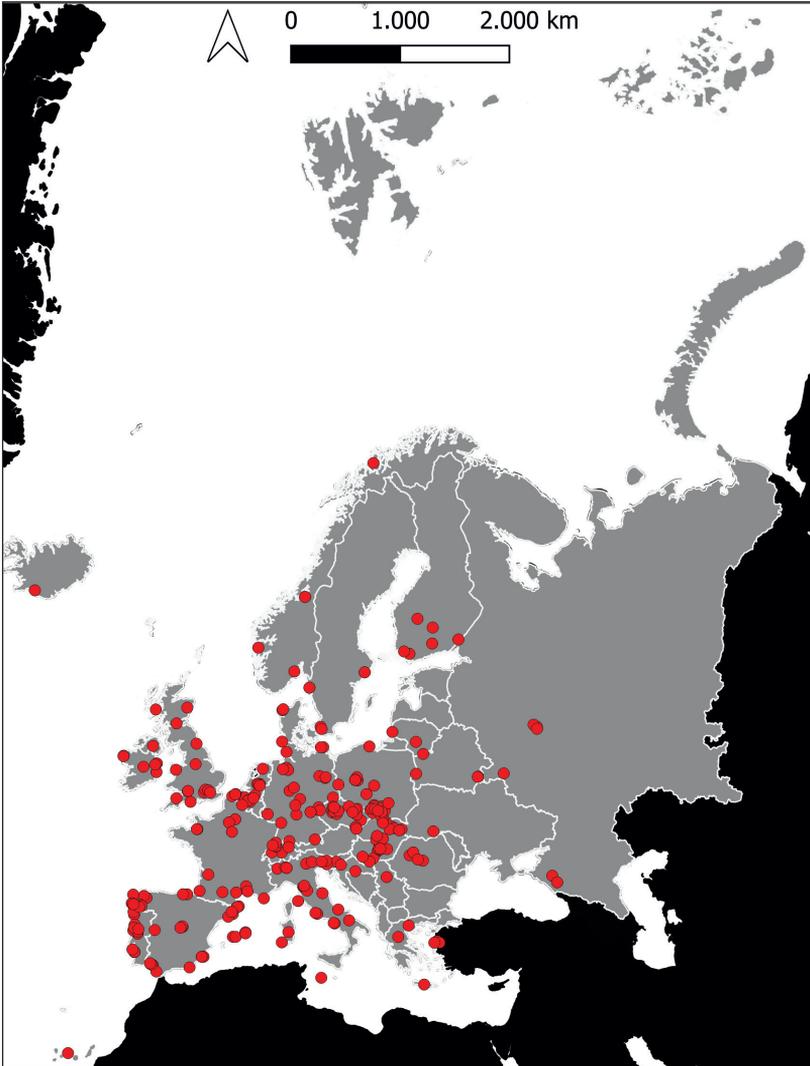


Figure 2. Map of locations (red dots) of field studies on the ecological impacts of invasive plant species in Europe. Twelve publications described studies in multiple countries and were represented by a dot in each country.

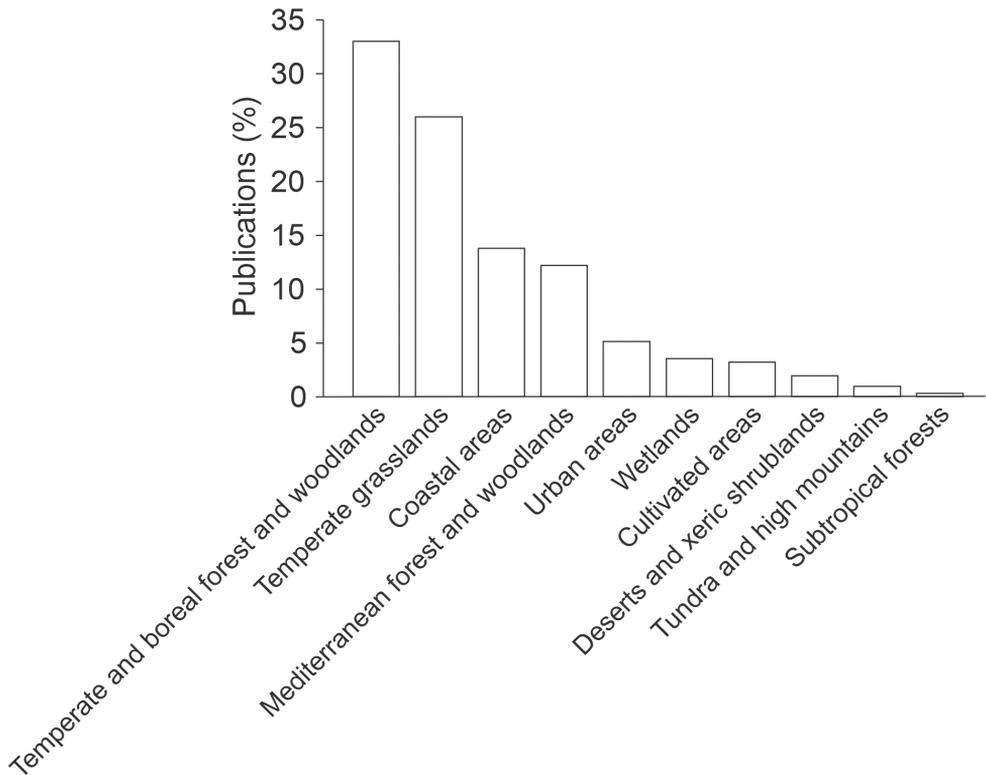


Figure 3. Percentage of publications on field studies testing for impacts classified by the invaded habitat in Europe. Habitats were classified according to the IPBES unit of analysis (IPBES 2018).

While the number of invasive plant species studied has increased linearly since about 2005, the number of publications on impacts have increased exponentially (Fig. 4a). One third of the publications examined the impacts of only five species (*Reynoutria japonica*, *Impatiens glandulifera*, *Solidago gigantea*, *Carpobrotus edulis* and *Robinia pseudoacacia*) out of 104 (Fig. 5). The studies on these five species have been concentrated in the last two decades and are still increasing to date (Fig. 4b).

The most studied impacts are on the abundance of species followed by impacts on the abundance and diversity of communities. Impacts on plants have been more studied than impacts on other taxa and trophic groups (Fig. 6). The second most studied impacted group is that of pollinators, followed by predators and decomposers (Fig. 6d). Impacts on microbial communities, although less frequently studied, have increased in the last few years (Fig. 6b). The number of field studies testing for impacts to ecosystem properties have increased one order of magnitude in the last decade (Fig. 4c), with impacts on nutrients pools and soil minerals being the most common (Fig. 6c).

Overall, 43% of studies found significant impacts of invasive plants with more decreases (26%) than increases (17%) on the response variables. Although more than half of the species (58 out of 104) have impacts in both directions, 10% of the invasive species showed only increase responses and 30% decrease responses (Fig. 5).

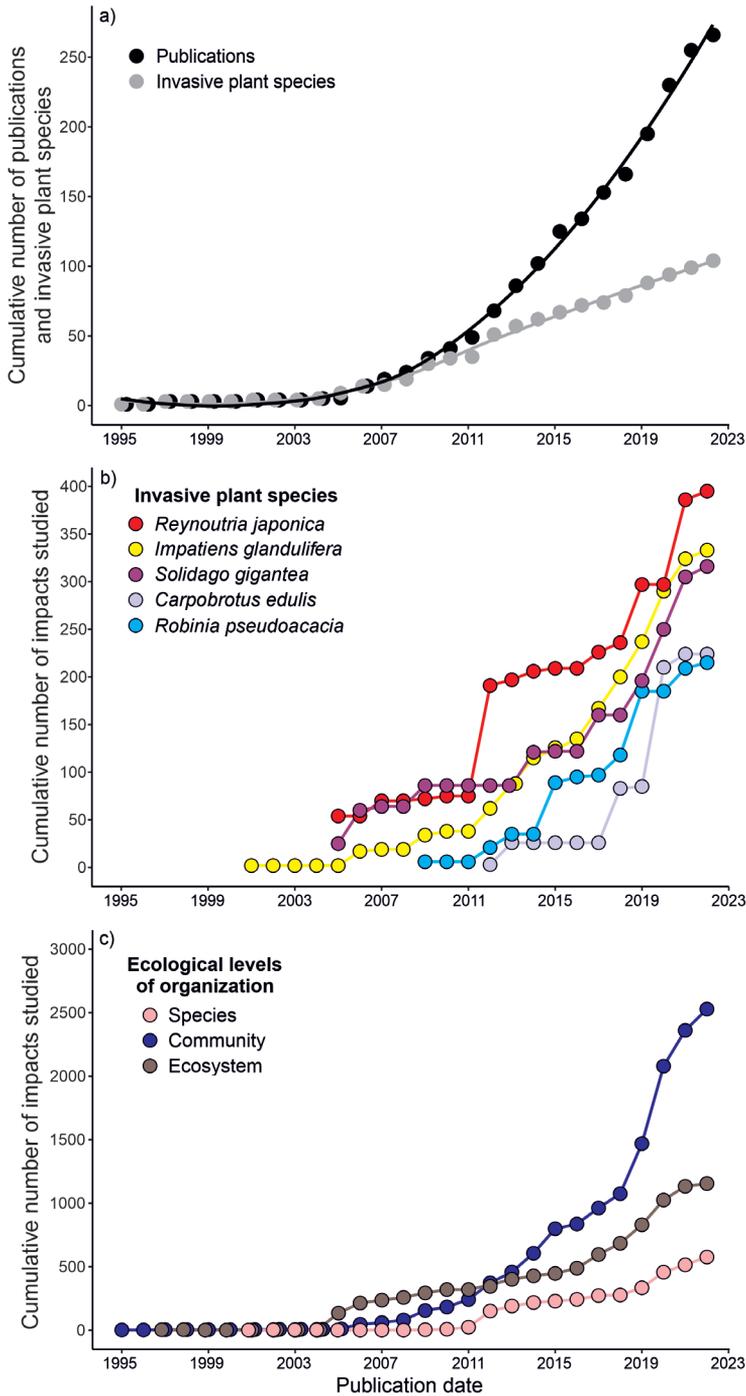


Figure 4. Cumulative total number of publications on impacts and invasive plant species studied (a), number of field studies testing for impacts on the five most studied species (b) and across ecological levels of organisation (c) in Europe. See Table 1 for impact type classification.

Invasive plant species	Studies	Publications	Frequency (%)	
<i>Reynoutria japonica</i>	395	19	39.2	
<i>Impatiens glandulifera</i>	333	35	40.2	
<i>Solidago gigantea</i>	316	21	39.6	
<i>Carpobrotus edulis</i>	224	9	45.1	
<i>Robinia pseudoacacia</i>	215	19	41.9	
<i>Prunus serotina</i>	206	12	33.5	
<i>Acacia longifolia</i>	191	13	66.5	
<i>Rosa rugosa</i>	170	7	38.8	
<i>Acacia dealbata</i>	158	9	50.0	
<i>Solidago canadensis</i>	126	14	41.3	
<i>Ailanthus altissima</i>	115	12	47.8	
<i>Lupinus polyphyllus</i>	100	11	41.0	
<i>Heracleum mantegazzianum</i>	96	8	30.2	
<i>Nicotiana glauca</i>	90	1	44.4	
<i>Asclepias syriaca</i>	76	8	13.2	
<i>Heracleum sosnowskyi</i>	76	4	63.2	
<i>Oxalis pes-caprae</i>	72	4	27.8	
<i>Pinus mugo</i>	53	7	71.7	
<i>Phytolacca americana</i>	51	1	35.3	
<i>Quercus rubra</i>	48	4	29.2	
<i>Amorpha fruticosa</i>	46	5	43.5	
<i>Senecio inaequidens</i>	43	3	37.2	
<i>Acacia melanoxylon</i>	43	1	60.5	
<i>Erigeron canadensis</i>	42	3	2.4	
<i>Reynoutria x bohemica</i>	42	4	59.5	
<i>Rhododendron ponticum</i>	41	7	36.6	
<i>Symphyotrichum lanceolatum</i>	40	3	40.0	
<i>Rudbeckia laciniata</i>	38	4	28.9	
<i>Impatiens parviflora</i>	35	6	40.0	
<i>Erigeron annuus</i>	29	2	6.9	
<i>Campylopus introflexus</i>	28	3	82.1	
<i>Prunus laurocerasus</i>	28	1	35.7	
<i>Symphyotrichum novi-belgii</i>	27	4	18.5	
<i>Sporobolus pumilus</i>	25	2	64.0	
<i>Solanum elaeagnifolium</i>	24	4	12.5	
<i>Arundo donax</i>	24	2	50.0	
<i>Genista aetnensis</i>	23	2	91.3	
<i>Acer negundo</i>	22	6	68.2	
<i>Carpobrotus acinaciformis</i>	20	5	45.0	
<i>Buddleja davidii</i>	20	3	30.0	

Figure 5. List of invasive plant species with the total number of field studies testing for impacts, publications and impact frequency (i.e. percentage of significant responses). Blue and orange bars indicate the proportion of decreases and increases, respectively.

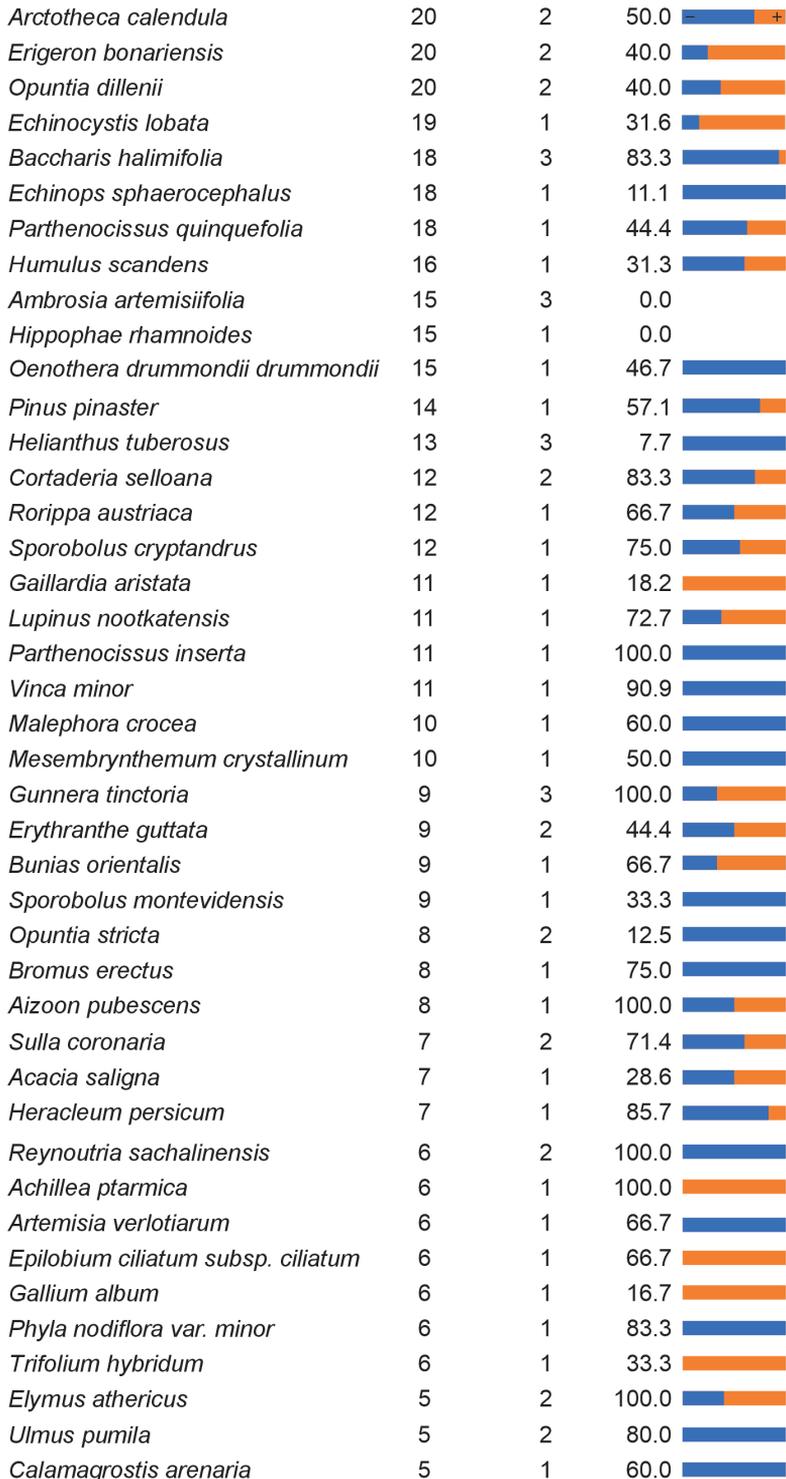


Figure 5. Continued.

<i>Acacia pycnantha</i>	4	1	100.0	
<i>Symphytotrichum × salignum</i>	4	1	100.0	
<i>Pteridium aquilinum</i>	4	1	50.0	
<i>Sorbaria sorbifolia</i>	4	1	75.0	
<i>Impatiens balfourii</i>	3	1	66.7	
<i>Ludwigia grandiflora</i>	3	1	33.3	
<i>Paspalum distichum</i>	3	1	33.3	
<i>Agave americana</i>	2	1	50.0	
<i>Bothriochloa ischaemum</i>	2	1	100.0	
<i>Calamagrostis epigejos</i>	2	1	100.0	
<i>Carpinus betulus</i>	2	1	100.0	
<i>Castanea sativa</i>	2	1	50.0	
<i>Imperatoria ostruthium</i>	2	1	50.0	
<i>Juglans regia</i>	2	1	100.0	
<i>Rumex alpinus</i>	2	1	100.0	
<i>Sporobolus anglicus</i>	2	1	100.0	
<i>Ficus carica</i>	1	1	100.0	
<i>Paspalum thunbergii</i>	1	1	0.0	
<i>Phacelia tanacetifolia</i>	1	1	100.0	
<i>Pinus sylvestris</i>	1	1	100.0	
<i>Silphium perfoliatum</i>	1	1	0.0	
<i>Xanthium orientale var. albinum</i>	1	1	0.0	

Figure 5. Continued.

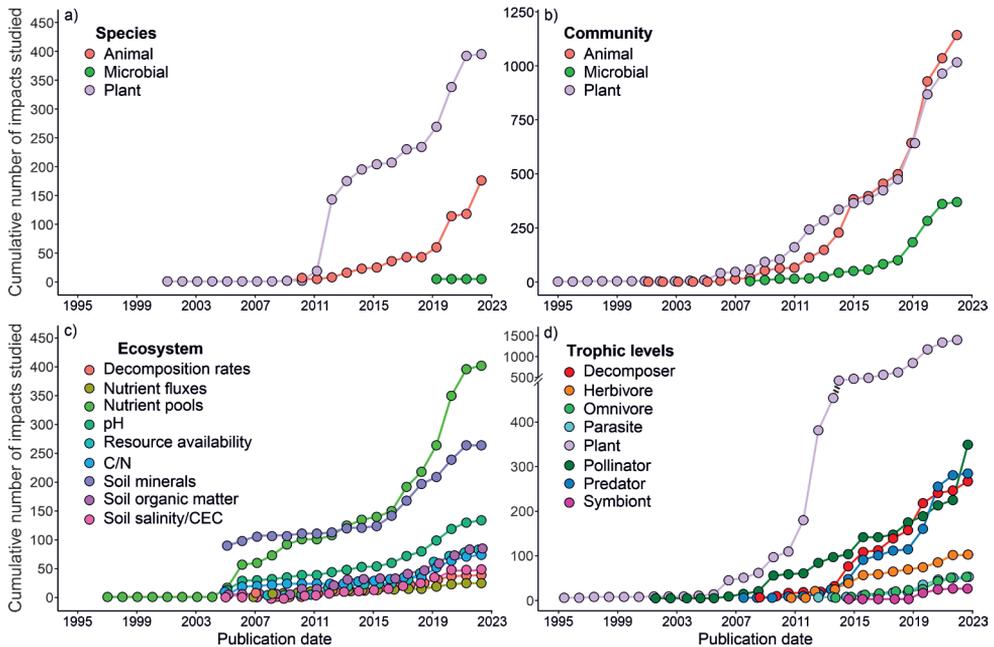


Figure 6. Cumulative number of field studies testing for impacts to species (a) and communities (b) by taxa, ecosystem properties (c) and amongst trophic levels (d) in Europe. See Tables 1, 2 for impact type classification.

Results on the frequency of significant impacts and their direction can be found in Fig. 7. In studies where the affected level is species, 41% of the impacts (n = 576) were significant, with more decreases (25%) than increases (16%) on the response variables. At the community level, 47% of impacts (n = 2528) were significant, with two times more decreases (32%) than increases (15%). At the ecosystem level, 38% of impacts (n = 1155) were significant, with fewer decreases (15%) than increases (23%). When impacts were classified by the affected trophic levels, altogether 45% of impacts (n = 2807) were significant, with two times more decreases (30%) than increases (15%).

The frequency of significant impacts was similar between the species and community levels ($z=0.17$, $p= 0.99$), but higher than at the ecosystem level ($z=2.32$, $p = 0.05$ and $z = 3.94$, $p < 0.001$, respectively). Additionally, the frequency of significant impacts was similar between animals and microbes ($z=0.17$, $p=0.99$), but lower than for plants ($z=3.86$, $p< 0.001$ and $z=2.94$, $p< 0.01$, respectively) (Fig. 8).

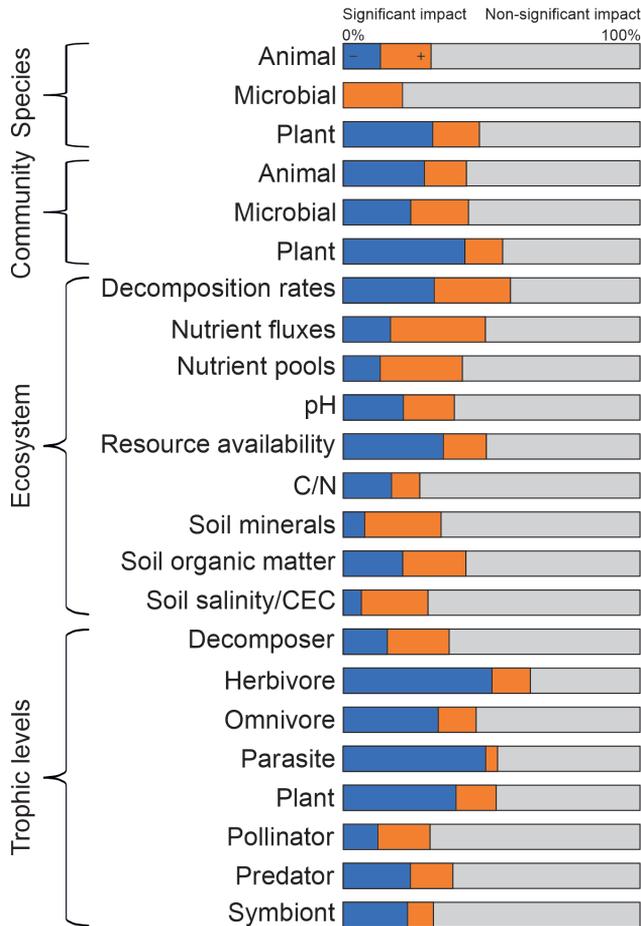


Figure 7. Frequency of significant plant invader impacts vs. percentage of non-significant impacts (grey bars) studied in field conditions in Europe. Blue and orange bars indicate the percentage of decreases and increases, respectively. See Tables 1, 2 for impact type classification.

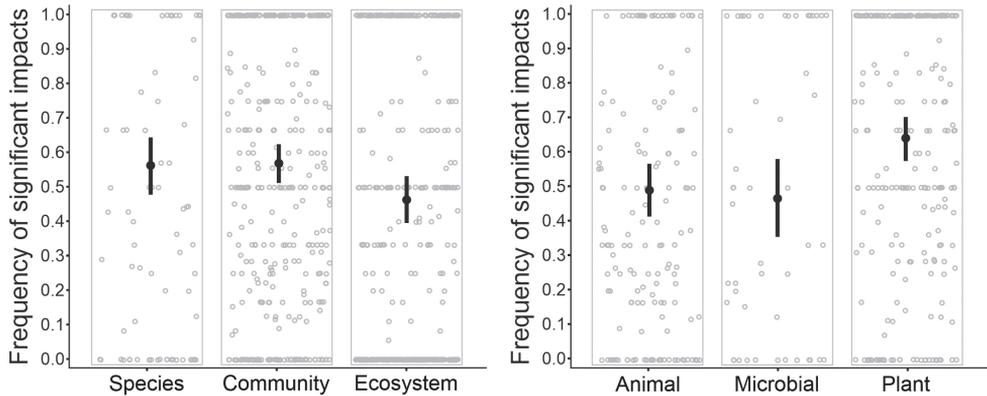


Figure 8. Frequency of significant plant invader impacts studied in field conditions in Europe across levels of ecological organisation (left) and taxa (right). Open circles are observed values (i.e. the proportion of significant impacts grouped by invader species and publication). Black dots are predicted values obtained from the models and their 95% confidence intervals.

Discussion

Evidence about the impacts of invasive plants on different properties of the recipient ecosystems is scattered across many different studies and technical reports (Kumschick et al. 2015), preventing its efficient transfer to managers and policy-makers. Here, we provide the first harmonised database synthesising results from field studies about the ecological impacts of invasive plants at a continental scale. However, a great proportion of studies focuses on a few invasive species in temperate central European countries or in southern Mediterranean countries. As already indicated ten years ago (Hulme et al. 2013), bias continues to be the norm in the study of impacts, probably reflecting the academic interest of research groups on the most common species in their countries. This database can be of scientific, management and policy use at different national and international scales.

The exploration of impact studies indicates that the main geographic gap of knowledge in Europe corresponds to Baltic and Balkan countries. The least represented habitats in impact studies are desert and xeric shrublands, high mountains and subtropical forests. In Europe, subtropical forests of major conservation status are located in Macaronesian islands, where non-native species invasion is prevalent. Many of these islands exhibit a higher proportion of non-native than native plant species in their flora (Kueffer et al. 2010), emphasising the crucial need to identify invasive species causing major impacts (Silva et al. 2008). The impacts of invasive plants in desert and semi-arid habitats are also poorly studied, despite an increasing number of dry-tolerant invasive plants promoted by ornamental xero-gardening (Morente-López et al. 2023). In the face of climate change, it is also imperative to focus more on the impacts of invasive plants in mountain regions. Climate warming is expected to enhance the dispersal and establishment of invasive species at higher altitudes, making this an area of critical concern (Carboni et al. 2018).

Ecological impacts were statistically heterogeneous in their significance and direction. Significant impacts were more frequent on species and communities than on ecosystems. Any change in ecosystem properties can be considered adverse, as it modifies ecosystem functioning (Strayer 2012; Vilà and Hulme 2017; Castro-Díez et al. 2019). Compared to impacts on ecosystems, the impacts on species and communities are more directly linked to changes in biodiversity. According to our database, there were two times more studies reporting negative effects than positive effects on the studied species and community response variables. Negative effects indicate a decrease in native species abundance, fitness or diversity after invasion and are, therefore, considered detrimental for nature conservation. On the contrary, positive effects indicate the reverse and, thus, can be assumed to be beneficial. However, even increasing effects on native species and communities can have cascading effects, depending on the position of the native species in the trophic network (Gallardo et al. 2016).

While the correspondence from value-free to value-laden effects of invaders on biodiversity is not always straightforward (Vimercati et al. 2020), our database on impacts studied in Europe contributes significantly to the global assessment on impacts of invasive species. This comprehensive database aligns with the broader finding that invasive species globally tend to cause more harm than benefits on nature (Bacher et al. 2023).

Significant impacts were more frequently reported on native plants than on native animals or microbes. In general, it seems that invasive species most frequently impact native species from the same broad taxonomic group (Bacher et al. 2023). For plants, this is an expected result because the main mechanism of interaction amongst plants is resource competition or facilitation, while the mechanisms of impact of invasive plants on animals are more diverse and often indirect, depending on the type of interaction, feeding mode and trophic position. Furthermore, impact studies on microbes are relatively recent and predominantly focused on soil bacteria and fungi (Dawson and Schrama 2016). However, it is important to note that, since our focus was on field studies, our review may not have captured all the impacts on plant-soil feedbacks, which are often mediated by microorganisms, such as pathogens or symbionts. It is largely unknown how the strength of plant-soil feedbacks compares with plant-plant competition. This is an area of research which deserves more attention because such interactions influence the co-occurrence of native and invasive species (Lekberg et al. 2018).

Other areas of research interest might include the analysis of the major causes of the variation in impacts and improving their prediction. For this purpose, the information provided in our database could be associated with other aspects of biological invasions (Strayer 2012). For instance, links with their pathways of introduction (Pergl et al. 2017), their local or regional abundances (Bradley et al. 2019), the species traits and the biotic and abiotic characteristics of the invaded habitats (Pyšek et al. 2012; Sapsford et al. 2020) or their invasion history (Lenzner et al. 2022). The frequency and direction of impacts could also be compared to those of invasive animals and amongst invaded areas to determine taxonomic differences in impact across regions.

Causal impacts, together with the probability of arrival and establishment, is one of the main requested information to identify potential invasive species. Therefore,

from a management point of view, the database displays and harmonises the available peer-reviewed publications that can be used for horizon scanning to identify potential invasive species in countries where they are not yet present (e.g. Lucy et al. (2020); Cano-Barbacid et al. (2023)). The information from the database can also be used to populate standardised impact assessments, such as the EICAT-IUCN (Blackburn et al. 2014) and to assist species management prioritisation, based on the magnitude of their impacts on biodiversity.

From a policy perspective, it is important to highlight that, although our analysis screened all European countries, the database does not include information for 29 of the 39 invasive plant species of EU concern (European Union 2014, 2017). Moreover, of the 20 most studied species according to our database, only three are regulated, namely *Impatiens glandulifera*, *Heracleum mantegazzianum* and *H. sosnowskyi*. These mismatches can be explained by some of the features of the EU Regulation (Carboneras et al. 2018). Some regulated species are not yet present in the EU (e.g. many aquatic plant species), but, if introduced, would be capable of establishing self-sustainable populations. On the other hand, some species are present, form self-sustainable populations and cause significant adverse impacts on biodiversity and ecosystem services in Europe, but listing the species will not prevent, minimise or mitigate their impacts and are therefore not listed.

Conclusion

Our first comprehensive European database of the field studies reporting on the ecological effects of invasive non-native plants indicates that invasive plants cause impacts to species, communities and ecosystem processes of a wide range of taxa at different trophic levels. Major gaps in knowledge are found in Baltic and Balkan countries, in desert and semi-arid shrublands, subtropical forests and high mountains. To improve the knowledge of the impacts of invasive plant species, we also advocate for more studies on species that are still locally rare and with restricted distribution, and on how they modify plant-soil-microbe interactions.

The information provided in this database is of interest for academic, management and policy-related purposes at the national, European and international scale. We acknowledge that our database may not encompass all relevant studies. The Web of Science has been the most widely used database for bibliometric analysis, offering more comprehensive coverage of older literature compared to Scopus. However, Scopus includes a larger list of journals than the Web of Science (Mongeon and Paul-Hus 2016). Further extensions of the database should also include a broader keywords string. For example, although restoration studies may not be explicitly designed to detect impacts, they can offer valuable insights into ecological differences between invaded plots before and after intervention in removal plots. Our database will have to be updated as new field studies on the ecological impacts of invasive species are published.

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Supplementary material I

Supplementary information

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Data type: docx

Explanation note: **table S1.** Definitions of the response variables used to classify impact types on native species (i.e. individuals of the same taxon) and communities (i.e. individuals of several species at a site). **table S2.** Publication level information in PLANTIMPACTSEUROPE_publicationLevel. xlsx. The PLANTIMPACTSEUROPE database can be accessed at <https://figshare.com/s/0a890d22bf5632fe5cb5>. **table S3.** Invasive plant information and field studies testing for impacts in PLANTIMPACTSEUROPE_impactsDatabase. xlsx. The PLANTIMPACTSEUROPE database can be accessed at <https://figshare.com/s/0a890d22bf5632fe5cb5>. **Storage location and medium:** The PLANTIMPACTSEUROPE database can be accessed at <https://figshare.com/s/0a890d22bf5632fe5cb5>. (1) PLANTIMPACTSEUROPE_publicationLevel. xlsx: 266 publications with indication of countries, habitats and study locations, 312 entries (rows excluding the header), 8 columns, 59 KB. (2) PLANTIMPACTSEUROPE_impactsDatabase. xlsx: 4259 impacts (rows excluding the header), 16 columns, 348 KB.

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Recent invasion of Ponto-Caspian amphipods in the Masurian Lakeland associated with human leisure activities

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Abstract

Non-indigenous species (NIS) contribute to the decrease of native species' diversity on a local and global scale. One of Europe's most significant donors of freshwater invasions is the Ponto-Caspian Region. Following the construction of artificial canals connecting isolated waterbodies and the resulting heavy boat traffic, the Ponto-Caspian Amphipoda started to spread in Europe. Four amphipod species: *Dikerogammarus haemobaphes*, *Dikerogammarus villosus*, *Pontogammarus robustoides* and *Chaetogammarus ischnus* have invaded the Masurian Lakeland (North-eastern Poland). Based on literature and our data, we studied their distribution in 22 lakes in the region during the years 2001–2016. We analysed their distribution against several water quality parameters and levels of anthropogenic pressure. Our results also present the first records of two new invaders, *D. villosus* and *C. ischnus*, in the studied area. We show that the relative abundance and frequency of these two species rapidly increase and, simultaneously, the populations of the earlier invaders, i.e. *D. haemobaphes* and *P. robustoides*, decrease. The native species – *Gammarus lacustris* – seems to be negatively affected by NIS richness, as well as by the proximity of towns. The spread of NIS in the lakes appears to be facilitated by boating and the lower complexity of the shoreline. Our study shows how anthropogenic pressure, especially tourism, can facilitate bioinvasion, jeopardising native biodiversity unless appropriate regulations are implemented.

Keywords

assemblage succession, biological invasions, lakes, propagule pressure, recreational boating, time series, tourist pressure

Introduction

Biological invasions are perceived as the second strongest threat to biodiversity on a local and global scale, right after habitat degradation (e.g. CBD (2000); Sala et al. (2000); Dudgeon et al. (2006); Kettunen et al. (2009); Strayer and Dudgeon (2010); Lambertini et al. (2011); Mačić et al. (2018); Lipták et al. (2019); Iqbal et al. (2021); Yang et al. (2021); Vantarová et al. (2023)). Many non-indigenous species (NIS) cause declines in abundance and diversity of native species, which is particularly apparent in Europe and the USA (e.g. Pinkster et al. (1992); Dick and Platvoet (1996, 2000); Ricciardi and MacIsaac (2000); Ricciardi (2006); Bellard et al. (2016); Panlasigui et al. (2018); Albano et al. (2021); Haubrock et al. (2021); Yang et al. (2021)) and is more prominent in freshwater ecosystems than in marine and terrestrial ones (Strayer and Dudgeon 2010). Many studies show high economic costs incurred by biological invasions on a global scale (Pyšek and Richardson 2010; Cuthbert et al. 2021a, b; Kouba et al. 2022). The average annual costs of preventing biological invasions and reversing their effects globally reach \$76 billion (Bradshaw et al. 2016); however, the costs of prevention of invasion are much lower than post-invasion management (Cuthbert et al. 2021a). Globally, the economic costs of aquatic bioinvasions have been estimated at \$23 billion (Cuthbert et al. 2021b). The costs of amphipod invasions constitute a small part of the global costs of aquatic crustacean invasions (\$180,000 out of an estimated \$271 million); however, these costs are underestimated (Kouba et al. 2022).

Even though surface freshwaters represent only 0.01% of the Earth's water resources and constitute 0.80% of the Earth's surface, they are inhabited by ca. 6% of the world's species (Dudgeon et al. 2006; Strayer and Dudgeon 2010). Therefore, freshwater ecosystems are precious from environmental, economic, sanitary, cultural and scientific perspectives and also constitute a valuable spot for tourism (Dudgeon et al. 2006; Hall and Härkönen 2006). Unfortunately, these ecosystems are in crisis, as indicated by stronger biodiversity loss than in terrestrial ecosystems (Dudgeon et al. 2006). According to the Water Framework Directive (European Environment Agency 2000), every waterbody in the EU should have achieved a high or at least good ecological and chemical status by 2015. However, the latest reports indicate that only 40% of such waters have achieved a satisfactory, healthy status (European Environment Agency 2018). Land use and agriculture are amongst the most important factors in aquatic ecosystems' declining conditions globally (Foley et al. 2005; Feld et al. 2016). Thus, although freshwater ecosystems constitute only a tiny fraction of the Earth's surface, high anthropogenic pressure results in a more pronounced negative impact of invaders on native species than in marine ecosystems (Ricciardi and Kipp 2008).

One of the richest European sources of species invading inland waters is the Ponto-Caspian Region (Ricciardi and MacIsaac 2000; Bij de Vaate et al. 2002; Galil et al. 2008; Panov et al. 2009; Copilaş-Ciocianu et al. 2023a). This region covers the coastal area of the Caspian, Black, Aral and Azov Seas, with their brackish limans and deltas of rivers discharging into these seas (Jażdżewski 1980). The Ponto-Caspian basin constitutes a hotspot of crustacean diversity, particularly in the case of amphi-

pod crustaceans (Cristescu and Hebert 2005; Väinölä et al. 2008; Copilaş-Ciocianu and Sidorov 2022; Copilaş-Ciocianu et al. 2022). Ponto-Caspian amphipods comprise around 10% of European freshwater invasive species (Pöckl et al. 2011). One of the main significant causes fuelling the bioinvasions of Ponto-Caspian species is the construction of canals that connect previously isolated watersheds (e.g. Jażdżewski (1980); Bij de Vaate et al. (2002); Nehring (2005); Galil et al. (2008); Arbačiauskas et al. (2010); Minchin et al. (2019); Jażdżewska et al. (2020)). Another important factor is translocations of species in ballast waters (Jażdżewski 1980; Pinkster et al. 1992; Bij de Vaate et al. 2002; Zhulidov et al. 2018). However, a more important vector of bioinvasions in freshwater ecosystems is transporting on biofouled hulls, filters and other submerged parts of vessels (Nehring 2005; Hewitt et al. 2009; Baćela-Spychalska et al. 2013; Anderson et al. 2014, 2015; De Ventura et al. 2016; Rewicz et al. 2017; Rodríguez-Rey et al. 2021). Biofouling of vessels by species resistant to desiccation enables their subsequent overland transport and the colonisation of isolated waterbodies (Baćela-Spychalska et al. 2013; Rachalewski et al. 2013; De Ventura et al. 2016). Fishing and diving equipment can also be an effective vector of invasions (Baćela-Spychalska et al. 2013; Anderson et al. 2014; Smith et al. 2020). Moreover, many species are also intentionally introduced into freshwater ecosystems (Grigorovich et al. 2002; Nehring 2005).

Seven species of Ponto-Caspian gammarids (Amphipoda, Gammaroidea) have already been recorded from Polish freshwaters: *Chaetogammarus ischnus* (Stebbing, 1899), *Dikerogammarus haemobaphes* (Eichwald, 1841), *Dikerogammarus villosus* (Sowinsky, 1894), *Obesogammarus crassus* (G.O. Sars, 1894), *Pontogammarus robustoides* (Sars, 1894), *Spirogammarus major* (Cărauşu, 1943) (former European population of *Echinogammarus trichiatus*) and *Chelicorophium curvispinum* (G.O. Sars, 1895) (Konopacka 1998; Gruszka 1999; Jażdżewski and Konopacka 2000; Konopacka and Jażdżewski 2002; Jażdżewski et al. 2005; Grabowski et al. 2007; Rachalewski et al. 2013; Copilaş-Ciocianu et al. 2023b). These species are already widely distributed in European inland waters, where they arrived through well-defined migration corridors: northern, central and southern (Bij de Vaate et al. 2002; Panov et al. 2009). Not only have they colonised the major rivers and canals constituting the invasions corridors, but also spread to the watersheds of these rivers, as well as many European lakes, for example, the Alpine Lakes (Rewicz et al. 2017) and the Great Masurian Lakes in Poland (Jażdżewski 2003; Jażdżewska and Jażdżewski 2008). An extensive up-to-date description of the distribution of alien freshwater amphipods in Europe can be found in Copilaş-Ciocianu et al. (2023a). As the dynamics of invasion in terms of species and ecosystem vulnerability varies and the impact of NIS depends on their invasion process (i.e. propagule pressure, species interactions), there is a constant need for monitoring and estimating trends and threats regarding invasions. The impact of invasive species on aquatic ecosystems is profound (Kurashov et al. 2012). Their introduction may lead to drastic changes in the macroinvertebrate community structure and affect the functioning of whole ecosystems (Jones et al. 1994; Jones et al. 1997; Lambertini et al. 2011). NIS can modify habitats as well as food chains and contribute to changes in

energy flows – benthic communities can be transformed from being energy suppliers to upper trophic levels becoming major consumers of ecosystem energy (Nalepa et al. 2009; Kurashov et al. 2012).

Lakes seem to be particularly susceptible to biological invasions, as many of them are under high tourist pressure, resulting in a higher probability of alien species introduction, even if the lakes are not directly connected with the invasion corridor (Bącela-Spychalska et al. 2013; Bącela-Spychalska 2016; De Ventura et al. 2016; Rewicz et al. 2017). One such region is the Masurian Lakeland. It is the most popular area for yachting in Poland and one of central Europe's main inland yachting regions. The region is extensively used for associated recreational activities, particularly angling and camping (Kistowski and Śleszyński 2010; Ulikowski et al. 2021). Unfortunately, the level of knowledge about the risks of spreading invasive Amphipoda in this region is poor and out of date (Jażdżewski 2003; Jażdżewska and Jażdżewski 2008). Previous studies were based on sampling from only a few lakes, provided mainly presence/absence data and predated the effect of increased recreational pressure. Knowledge about the role of tourism, shipping and other factors in biological invasions in the Masurian Lakeland is poor and demands study. Given the significance of these factors in other regions, it is likely that their influence in the Masurian Lakeland is also considerable. The intensity of shipping and, therefore, its effect on biological invasions will increase with time (Sardain et al. 2019). Thus, it is crucial to understand these mechanisms in the study area. We also do not know how the invasion of amphipods affected native amphipods in the region. With regards to the faunistic data about the native amphipod species in the Lakeland, Jażdżewski and Konopacka (1995) mention two widely distributed lacustrine species, namely *Gammarus lacustris* G.O. Sars, 1863 and *Pallasiola quadrispinosa* (G.O. Sars, 1867). However, these data are old and require updating.

The aims of our study were: i) to update the knowledge on the distribution and expansion of the Ponto-Caspian amphipod fauna in the Masurian Lakeland; ii) to assess the distribution of native vs. invasive Ponto-Caspian amphipods in the context of biotic and abiotic characteristics of the lakes and anthropogenic pressure in this region, using both historical and newly-obtained data. Based on observed trends in other regions (e.g. Dick and Platvoet (2000); Grabowski et al. (2006); Van der Velde et al. (2009); Meßner and Zettler (2021)), we assumed that some invasive amphipods are replaced by stronger competitors and that native species are not able to co-exist with the invasive species. We hypothesise that high tourist pressure contributes to the dispersion of invasive amphipods, while the occurrence of the native species is linked to isolated lakes.

We tracked the distribution of invasive Amphipoda in the Masurian Lakeland since 2001, based on literature and our data. To explore the relationship between the structure of amphipod assemblages and lake characteristics, including human tourist pressure in the years 2014 and 2016, we collected data on the relative abundance of amphipods, measured basic water parameters, implemented hydromorphological data and estimated the tourist pressure.

Materials and methods

Study area

The Masurian Lakeland (Pojezierze Mazurskie in Polish) is a lake district (macroregion) in North-eastern Poland with a surface area of 52,000 km² including seven mesoregions, amongst others, the Land of the Great Masurian Lakes (Kraina Wielkich Jezior Mazurskich in Polish) and the Ełckie Lakeland (Pojezierze Ełckie in Polish) (Kondracki 2002). The landscape was formed between 16,000 and 11,000 BP (at the end of the last glaciation) and is characterised by strong latitude differentiation, dominantly with moraine hills (Hillbricht-Ilkowska et al. 2000; Ulikowski et al. 2021) and with glacial tills as a dominant component of the soil substratum (Hillbricht-Ilkowska et al. 2000). The lakes are mainly surrounded by a mosaic of agricultural areas and forests giving similar input of allochthonous organic and mineral matter to each lake (Chróst and Siuda 2006; Ejsmont-Karabin et al. 2020). Most lakes of this region are dimictic with summer thermal stratification (Ulikowski et al. 2021). They are connected with main European watersheds via artificial canals and small rivers: the River Pisa (flowing into the River Narew and then into the River Vistula) and the River Węgorapa (flowing into the River Pregolya and then into the Vistula Lagoon) (Bajkiewicz-Grabowska 2008; Jażdżewska and Jażdżewski 2008; Ulikowski et al. 2021). This connectivity increases the probability of invasive amphipods spreading in the region. For this study, we selected lakes with historical faunistic data, based on Jażdżewski and Konopacka (1995), as well as along a gradient of tourist pressure, including more natural and isolated lakes. We also selected sampling points on the rivers, i.e. the River Węgorapa, the River Pisa and the River Narew, which connect the Masurian Lakeland with major rivers, for example, the River Vistula and the River Neman (Fig. 1; see also Suppl. material 1).

Sampling and data collection

Our dataset consists of two types of data: (i) published, including the years between 2001 and 2007 (Jażdżewski 2003; Jażdżewska and Jażdżewski 2008) and (ii) new data coming from field surveys in 2008, 2009, 2014 and 2016. Additionally, to facilitate the monitoring of the amphipod expansion and to model the distribution of native *Gammarus lacustris*, we incorporated records from several lakes and the River Narew, which are situated outside of the study area (see Suppl. material 1). The studies that were conducted between 2001 and 2009 only have a qualitative character (i.e. presence/absence of amphipod species), while for 2014 and 2016, the species abundances are available. Generally, sampling was done through “kick-sampling” with a benthic hand-net with a mesh size of 0.5 mm, used for 45 min at each station, performed by two people with equal effort, from all available littoral habitats (sand, mud, gravel, stones and submerged macrophytes) at depths from 0.05 to 0.5 m. Such a semi-quantitative method gives reliable and comparable results for all sampling points and all study years/periods (Jażdżewski et al. 2002; Grabowski et al. 2006). The amphipods

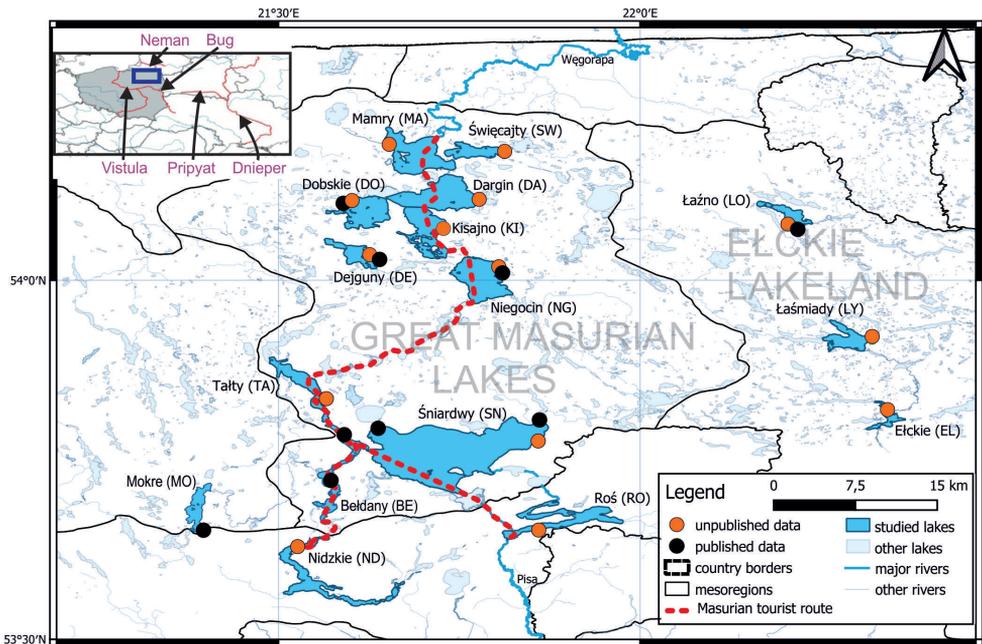


Figure 1. The sites in the Masurian Lakeland. Sites were divided into previously unpublished (records of this study) and published (Jażdżewski 2003; Jażdżewska and Jażdżewski 2008). Mesoregions are delimited according to Kondracki (2002). The two-letter acronyms for particular lakes were used in further Figures and Suppl. material 1.

were preserved in 96% ethanol and then identified in the laboratory to the species level, based on the available literature (Mordukhai-Boltovskoi 1964; Eggers and Martens 2001). This collection and preservation protocol was used at all studied sites and in all study years.

To detect the potential role of biotic and abiotic factors, as well as human pressure on the presence of invasive amphipods in the lakes sampled in 2014 and 2016, we used topological and anthropogenic variables, such as the surface-volume ratio or the distance from town. As a proxy of the level of anthropogenic pressure, we used the water quality status (water QS) from Soszka et al. (2016). This index categorises the waterbodies into six water quality categories (ranging from excellent – class I, to very poor – class VI), based on species assemblages and chemical and physical parameters of water according to the Water Framework Directive (European Environment Agency 2000). We presume that lower values of this variable (lower water class), indicating increased species diversity and reduced levels of nutrients and heavy metals in the water (better water quality), correspond to lower levels of anthropogenic pressure on the lake (European Environment Agency 2000; Sánchez et al. 2007; Lobato et al. 2015). Environmental heterogeneity creates more niches that can be occupied by co-occurring species (Chesson 2000). Thus, we used two indices: shoreline development (shoreline length to surface area ratio) from *mojemazury.pl* and surface

area to volume ratio (A/V ratio) from Soszka et al. (2016). The shoreline development index is the ratio of the actual shoreline length of a lake to the circumference of a perfectly circular lake with the same area (Aronow 1982). High values indicate a more complex shoreline, retaining a higher load of nutrients from land (Cole 1975) and providing more niches for the biota (Chesson 2000). The surface area to volume ratio combines information about the depth and size of the lake and can be positively correlated with the productivity of the lake (Fee 1979). Smaller waterbodies (lower A/V ratio) may play the role of refugia for native species (Grabowski et al. 2009). The density of boats (i.e. the number of boats divided by the lake surface in ha), was obtained as the maximum possible number of moored boats in marinas (Johnson and Padilla 1996; Vander Zanden and Olden 2008; Ros et al. 2013). We assumed that the higher the density of boats in the lakes, the higher the tourist pressure and the higher the probability of transport of invasive species by vessels (Johnson and Padilla 1996; Vander Zanden and Olden 2008; Bączela-Spychalska et al. 2013; Ros et al. 2013). The maximum capacity of marinas was obtained from websites: mazury24.eu and skorupki.mazury.info.pl. Tourist infrastructure is mainly localised in urban areas (Kulczyk et al. 2016). Thus, we used the distance between the sampling point and towns (i.e. centroid) as an estimation of anthropogenic pressure. Moreover, land use in the vicinity of water-bodies can impact the temporal variations in amphipod assemblages (Cereghetti 2023). The distance was measured as a linear distance in km from the centroid of the closest town to the sampling point using QGIS software. Towns were designated according to the ESRI shapefile “UIA World Countries Boundaries”, available at: <https://hub.arcgis.com/datasets/UIA::uia-world-countries-boundaries>. All spatial analyses and their visualisation were conducted using QGIS 3.10.13 (QGIS Development Team 2020).

Data analysis

Using all unpublished records since 2008 from the lakes and the rivers, including sites outside the study area (see Suppl. material 1), we modelled the presence of the only native gammarid (i.e. *Gammarus lacustris*) according to the number of NIS and the relative distance of each sampling site from town. We included this variable as a proxy of the anthropogenic propagule pressure (i.e. the introduction of NIS by human activities) of NIS at each site (i.e. inversely correlated). We used generalised linear mixed models (GLMMs) to include the random variable of the sampling year. Given the presence/absence nature of the data, we used a Bernoulli distribution fitted with glmmTMB (link = logit) with the homonymous package (Brooks et al. 2017). The possible inclusion of the interaction between NIS richness (i.e. number of species) and the distance from the closest town was also tested using the Akaike Information Criterion (AIC; Bozdogan (1987)). After fitting the model, we validated it by simulating its residuals using the package DHARMA (Hartig 2022). We also confirmed the absence of spatial autocorrelation of the residuals using the Spatial Autocorrelation function of the DHARMA package.

Using samples collected in 2014 and 2016, we first explored the variability of the environmental parameters of the sites and lakes, grouping them according to their geographical position and connectivity (i.e. I: northern, II: southern, III: eastern; Fig. 2B, see also Suppl. material 1). We hypothesised that nearby and interconnected lakes would exhibit comparable gammarid assemblages. This assumption is supported by findings from the Great Lakes in the USA, where the likelihood of species invasion was found to be the highest near the mouth of canals connecting the lakes (Grigorovich et al. 2005). To explore and visualise the environmental variability of the study area, we used a principal component analysis (PCA) with standardised values with *prcomp* of the package *vegan* (Oksanen et al. 2022). We analysed the gammarid assemblage using a permutational multivariate analysis of the covariance (PERMANCOVA) with an orthogonal design with two fixed factors (i.e. lake groups with three levels – I, II, III; time with two levels – 2014 and 2016) and five covariates: water QS, A/V ratio, shoreline development, density of boats and distance from the town. To control the possible sampling differences (i.e. being semi-quantitative), Hellinger distances were used to compare the abundances of the different species. To account for the excess of zero values, a dummy variable of 0.0001 was added to the whole dataset. We used *first adonis2* of the package *vegan* with 9999 permutations and *pairwise.adonis* of the package *pairwiseAdonis*, with Holm correction and 9999 permutations, for the post hoc analysis between levels of the significant factors (Martinez Arbizu 2020). To visualise and corroborate the results of the PERMANCOVA, we finally used a constrained ordination using distance-based redundancy analysis (dbRDA), based on Legendre and Anderson (1999), with *capscale* (package *vegan*) and Hellinger distances, as for *Permancova*, including the covariates of the PERMANCOVA as constraining variables. All the analyses were performed in the R environment 4.3.0 version (R Core Team 2023).

Results

Temporal and spatial distribution of invasive species

We recorded four invasive gammarid species from 12 lakes and the Rivers Węgorapa and Pisa and one native species (*Gammarus lacustris*) from 16 lakes (Fig. 2A, Suppl. material 1). The first recorded invasive species was *Dikerogammarus haemobaphes* found in 2001 (Jajdzewski 2003) and the second was *Pontogammarus robustoides*, which was first observed in 2007 (Jajdzewska and Jajdzewski 2008). The spread of invasive species can be observed over time (Fig. 2B). Between 2014 and 2016, *D. haemobaphes* spread to one more lake and is observed now in nine of them. *Pontogammarus robustoides* did not colonise new lakes in 2016, compared to 2014. In 2014, we noticed the first appearance of the other two invaders: *C. ischnus* and *D. villosus* (Fig. 2A). The previous species was found in two lakes in 2014 and expanded to five further lakes in 2016, while the latter one was already found in five lakes in 2014 and expanded to two further lakes in 2016 (Fig. 2A). Although *Chaetogammarus ischnus* was recorded in the River Pisa in 2014 and 2016, *D. villosus* was not found in any of the studied rivers

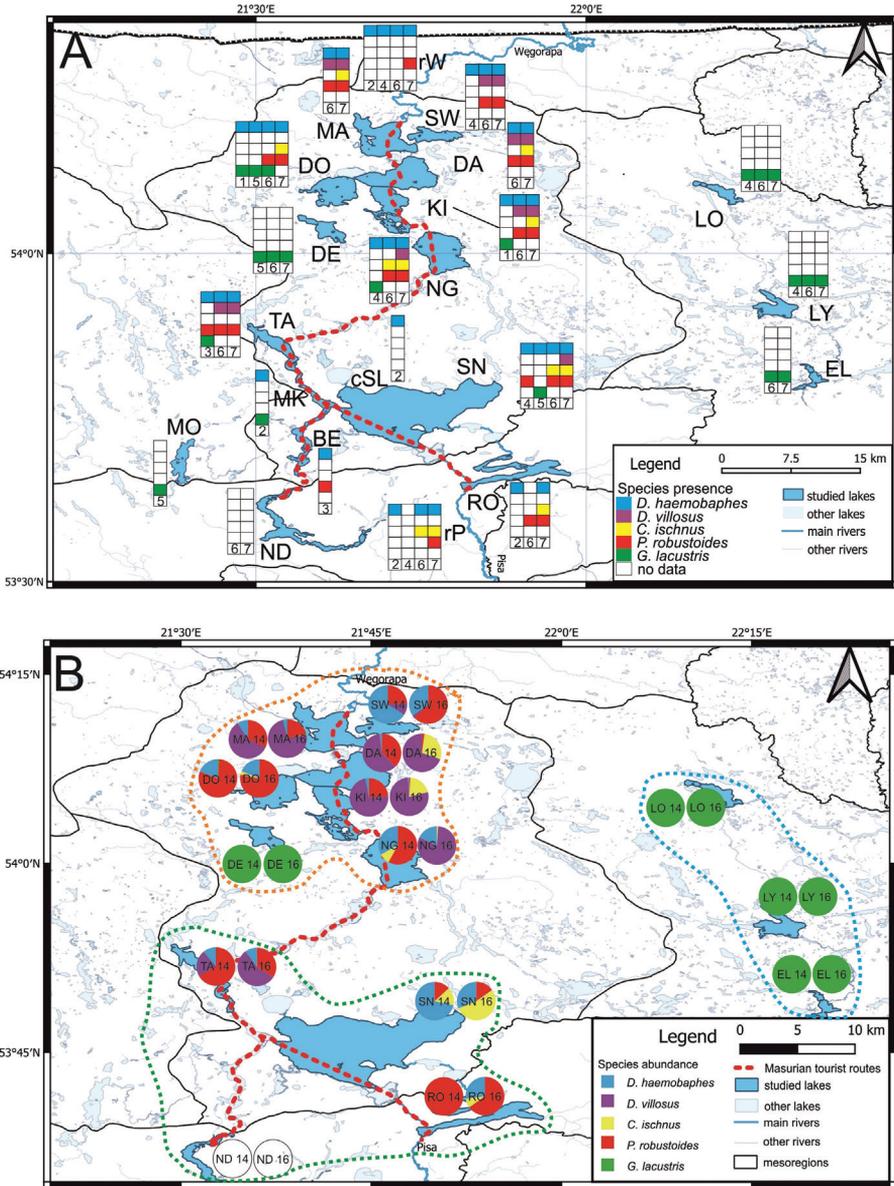


Figure 2. **A** the distribution of invasive and native amphipod species in studied lakes since 2001, based on published and new data (locality codes according to Suppl. material 1). Table at each lake showing the assemblage (colours in rectangles according to different species, see legend) variation in time (symbols for sampling years: 1 – 2001; 2 – 2002; 3 – 2007; 4 – 2008; 5 – 2009; 6 – 2014; 7 – 2016). Only years of samplings from each lake and river are shown. Colourless rectangles indicate that no amphipods were recorded during the sampling. The dashed black line indicates country borders; the dashed red line indicates the Masurian tourist boat route. Black lines delimit mesoregions according to Kondracki (2002) **B** the assemblage composition of the amphipod fauna in studied lakes in the years 2014 and 2016 (locality codes according to Suppl. material 1). Pie charts show the relative abundances of each species. An empty circle means no amphipods were recorded. Black lines delimit mesoregions according to Kondracki (2002). Coloured dotted lines around the pie charts correspond to the lake groups: orange – I, green – II, blue – III.

(Fig. 2A) The relative abundance of new invaders (*D. villosus* and *C. ischnus*) increased with time, while it decreased for *D. haemobaphes* and *P. robustoides* (Fig. 2B). In Lake Nidzkie, we did not record any amphipod species (Fig. 2A, B).

The modelled occurrence of native *Gammarus lacustris*

Generally, the native species – *Gammarus lacustris* – was not found in lakes inhabited by invasive species, apart from Lake Dobskie, where the native and invasive gammarids co-occurred in 2014 with a low number of *G. lacustris* (two individuals vs. 194 individuals of invasive species) (Fig. 2A, B; Suppl. material 1). The GLMM for the presence of *G. lacustris* showed the significant negative effect of NIS richness (p-value = 0.002) and the positive effect of the distance from town (p-value = 0.024), but not their interaction (Fig. 3). The inclusion of the year as a random effect barely increased the R^2 (Marginal 0.733 – Conditional 0.808), supporting the effectiveness in sampling efforts (Suppl. materials 2, 4).

Environmental factors and amphipod assemblage

The first three components of the PCA explained 85.5% of the variance amongst the environmental variables (Fig. 4A, B). According to PC1 and PC3 (~ 57% variance explained), the lakes further from the tourist route (i.e. group III) are, indeed, characterised by a lower number of boats, higher complexity of the shore and a greater distance from town. The PC2 was more related to the water quality status (water class) and the surface-volume ratio showing a general trend of better water quality (lower class of water quality status) and deeper waters for group I (highest class of water status – lowest water quality for group II). The PERMANCOVA results showed significant effects (p-values < 0.05) of shoreline development ($F = 22.096$, $p < 0.001$), the number of boats ($F = 10.788$, $p < 0.001$) and water quality status – water class ($F = 3.794$, $p = 0.035$) on the assemblage of amphipods (Suppl. material 3). Even though the relative abundance of species changed with time, i.e. increased in *D. villosus* and *C. ischnus* and decreased

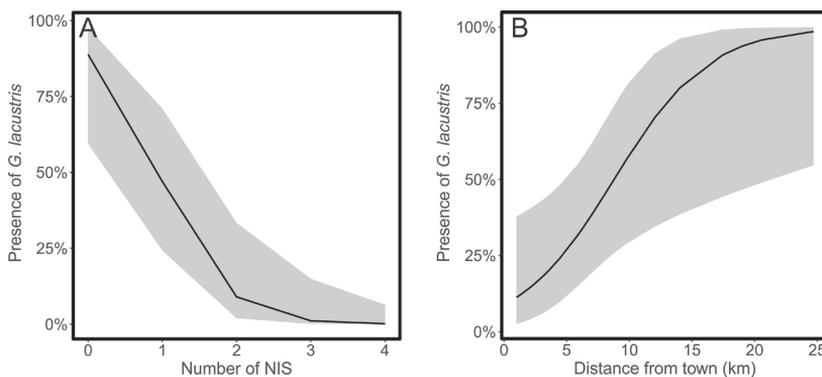


Figure 3. The predicted probability of occurrence of *G. lacustris* dependent on the richness of NIS (A) and the distance of the sampling point from town (B). The grey area delimits the 95% confidence intervals.

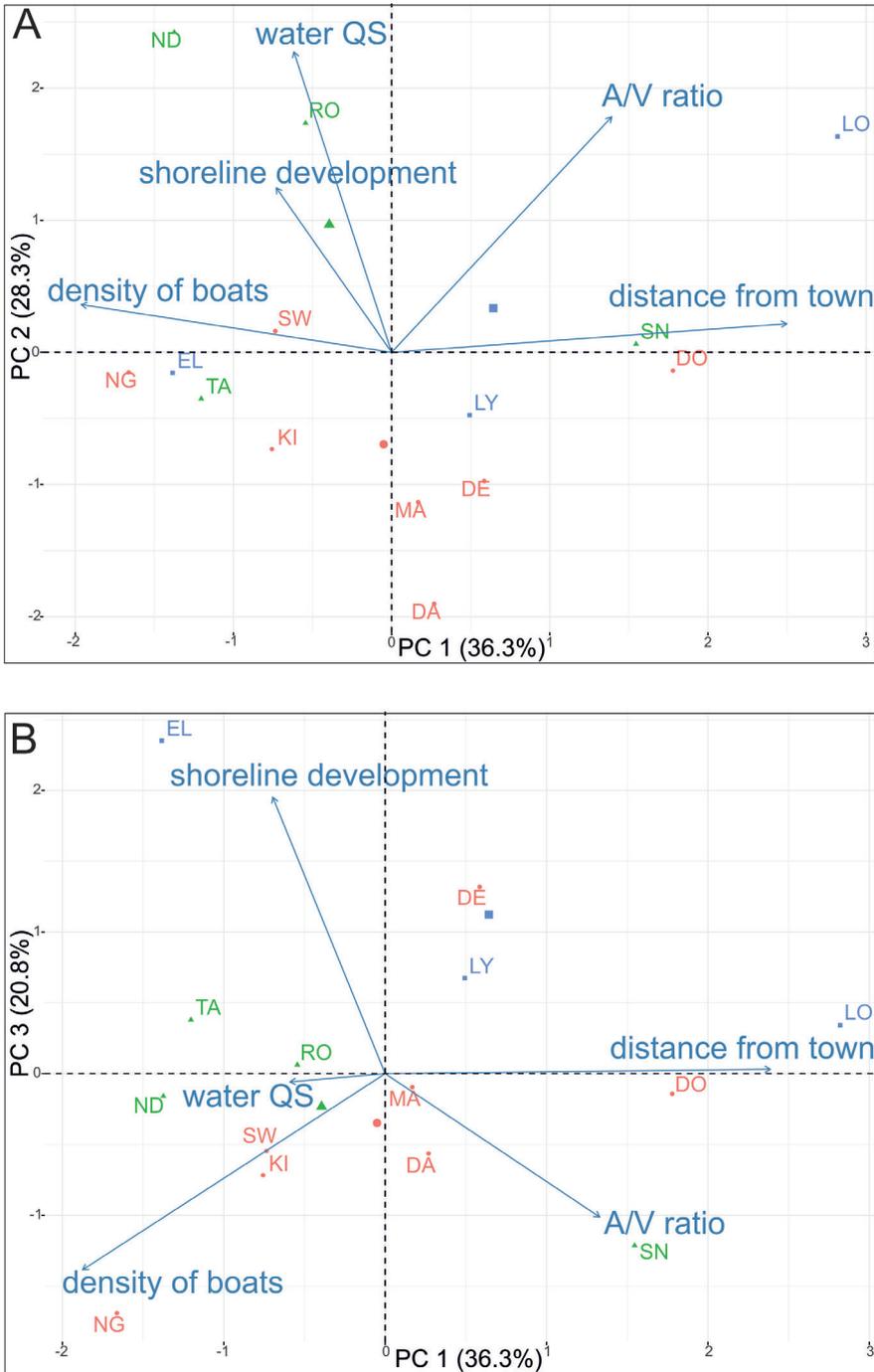


Figure 4. Biplots displaying the first three axes of the PCA of the environmental variables of the lakes sampled in 2014 and 2016 (**A** PC1-2 **B** PC1-3). The colours refer to the different lake groups: orange circles (I), green triangles (II) and blue squares (III). The lengths of the arrows are proportional to the loading of each variable, dashed lines = 0. The acronyms of lakes are according to Fig. 1 and Suppl. material 1.

in *D. haemobaphes*, *P. robustoides* and *G. lacustris*, the time factor was not significant. The differences in amphipod assemblages between lake groups (determined, based on the geographical position and interconnections between the lakes) were marginally significant, i.e. $F = 2.680$, $p = 0.057$) and the post hoc analysis showed a significant difference ($p_{\text{adjusted}} < 0.001$) between the group III (i.e. eastern group) and the others, but not between the first two ($p_{\text{adjusted}} > 0.4$).

The first two axes of the dbRDA fitted 90.1% of 52.1% of the total variation explained (Fig. 5). The presence of the native *G. lacustris* appeared more correlated to lakes with more complex shorelines. The occurrence of *D. villosus* was mainly explained by the increasing number of boats and proximity to town. The other three species (i.e. *P. robustoides*, *D. haemobaphes* and *C. ischnus*) seemed to be related to simpler shorelines and average values for the other variables, which was generally the opposite to *D. villosus*.

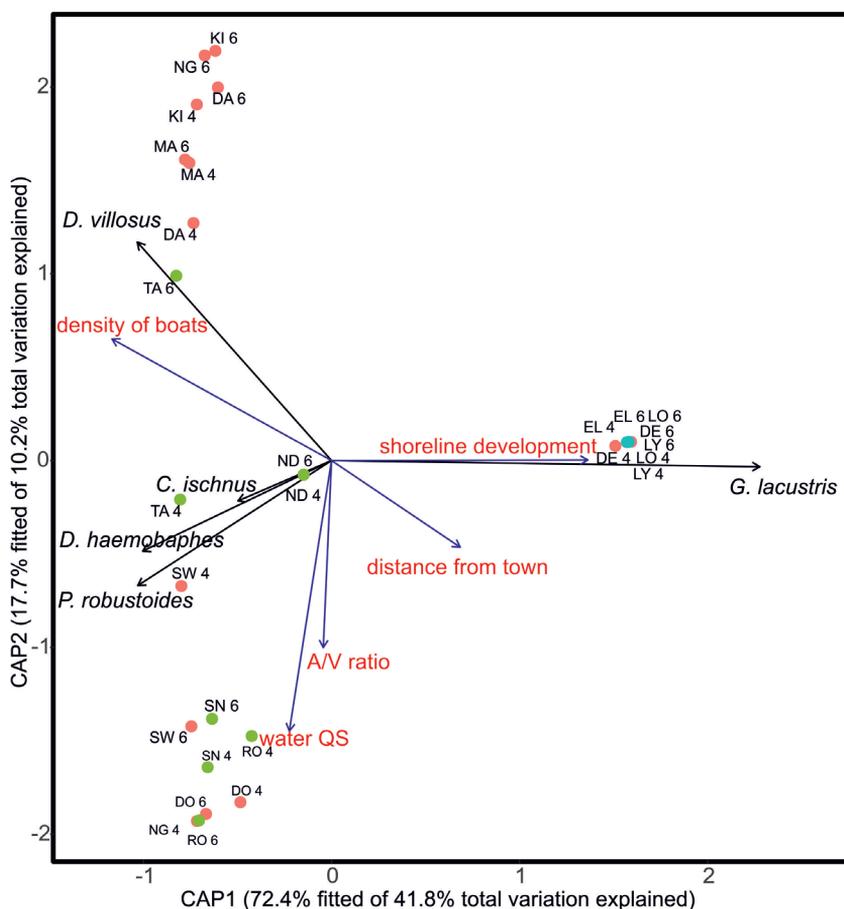


Figure 5. Canonical analysis of principal coordinates (CAPSCALE) derived from the Bray-Curtis dissimilarities of the gammarid assemblages and the environmental variables of the studied lakes in the years 2014 and 2016. The colours of the dots refer to the different lake groups: orange circles (I), green circles (II) and blue circles (III).

Discussion

Our study shows that, between 2001 and 2016, the number of invasive amphipod species in the study area increased drastically from one (*D. haemobaphes*) to four (three more species recorded: *D. villosus*, *P. robustoides*, *C. ischnus*). Simultaneously, a continuous decrease in the occurrence of native *Gammarus lacustris* was recorded. Our study reveals that the presence of NIS in lakes is primarily facilitated by three key factors: recreational boating activities, proximity to urban areas and simplified lake shorelines.

Distribution of *Gammarus lacustris*

According to our results, the presence of more than one NIS significantly affects the presence of the native *G. lacustris*, bringing the probability of its presence almost to zero already with three NIS (Fig. 3A). The species disappeared several years after the expansion of invasive amphipod species in several lakes (Fig. 2A, Suppl. material 1). For instance, the species was widely distributed until the last record in 2001 in Lake Kisajno (Jażdżewski 2003), in 2007 in Lake Tałty (Jażdżewska and Jażdżewski 2008), in 2008 in Lake Niegocin and in 2009 in Lake Śniardwy. Older data mention the presence of *Gammarus lacustris* in Lake Mamry (Jażdżewski 1975). In these lakes, the disappearance of *G. lacustris* coincided with the invasion of alien species. In 2014, *G. lacustris* was co-occurring with invasive species in only one lake (Lake Dobskie). One potential explanation could be the limited tourist activity in Lake Dobskie, as well as low species introduction probabilities, resulting from the absence of direct connections between this lake and other lakes situated along the Masurian tourist routes. Moreover, in 2014, the invasion of *C. ischnus* and *P. robustoides* in Lake Dobskie was still in its early stage. However, in 2014 the abundance of *G. lacustris* in this lake was very low and we did not record this species in 2016. Additionally, in 2002, we recorded the species co-occurring with *D. haemobaphes* in Lake Mikołajskie, but the presence of *G. lacustris* in this lake in subsequent years is unknown. In general, most of the records of *G. lacustris* in the Masurian Lakeland come from isolated lakes where invasive amphipods did not spread. In 2016, we found this species only in four isolated lakes, i.e. Dejguny, Elckie, Łaśmiady and Łażno (Fig. 2A; Suppl. material 1).

These four lakes (three of them in the eastern group of lakes) are characterised by low tourist pressure (low number of boats, long distance from the tourist routes) (Fig. 5). The low level of tourist pressure in these lakes and lack of direct connections with the Great Masurian Lakes (central part of the Masurian Lakeland), where all the invasive amphipods are present, may create a refuge for native species. Furthermore, we found that *G. lacustris* is associated with lakes distanced from towns (Figs 3B, 5). The proximity of the lakes to the urban areas results in their pollution and declining quality of water (Mishra et al. 2023). Although *G. lacustris* has a broad tolerance to environmental factors (Matafonov and Bazova 2014), its populations decline in polluted water, for example, with high acidity (Okland 1969) and pesticides (Gerhardt et al. 2011). Hence, it can be anticipated that *G. lacustris* will primarily be distributed in

lakes with low anthropogenic pressure. Our results of CAPSCALE analysis show that higher classes of water quality status (lower water quality) characterise mainly lakes inhabited by *C. ischnus*, *D. haemobaphes* and *P. robustoides* (e.g. Lake Śniardwy, Lake Roś, Lake Święcajty), where we did not record *G. lacustris* (apart from Lake Śniardwy in 2009) (Fig. 5).

Instead, we recorded *G. lacustris* in lakes characterised by a high level of shoreline development. Lakes with higher shoreline complexity may provide higher habitat diversity, resulting in lower competition rates between species on environmental resources and, consequently, promoting the possible co-existence of many species, both native and invasive amphipods (Chesson 2000; Amarasekare 2003). While in the lakes with the lower value of this index, native amphipods may be unable to compete with invasive species and could become extinct. However, our results do not confirm this assumption. We found *G. lacustris* in lakes with high shoreline development (e.g. Lake Ełckie), but no invasive amphipods were found there. Isolation of these lakes and low tourist pressure could result in the lack of conditions for their invasion. Nonetheless, in the event of their invasion, we can suppose that the high shoreline complexity of these lakes would promote the co-existence of native and invasive amphipods.

The declining populations of *G. lacustris* in our studies are similar to the general tendency observed in Europe. This species seems to be one of the weakest competitors amongst European freshwater amphipods giving way to the Ponto-Caspian species of genera: *Chaetogammarus*, *Dikerogammarus* and *Pontogammarus* (Meßner and Zettler 2021). *Gammarus lacustris* occurs in a wide range of habitats; nevertheless, in the last few decades, the species has been pushed to the relict range of occurrence (Hesselschwerdt et al. 2008; Meßner and Zettler 2021). Nowadays, the species is present almost exclusively in isolated waterbodies and continues to decline (Meßner and Zettler 2021). The population decline is also attributed to the hydromorphological and hydrochemical changes that occur in aquatic ecosystems (Okland 1969; Matafonov and Bazova 2014).

Similarly, we did not record another native amphipod, *Pallasiola quadrispinosa*, also recorded as declining in the freshwater ecosystems due to invasive amphipods (Żmudziński 1995; Jażdżewski et al. 2004). According to Jażdżewski and Konopacka (1995), this species was found in several lakes of the Masurian Lakeland, i.e. Dargin, Dobskie, Ełckie, Kisajno, Łaśniady, Mamry, Mikołajskie, Mokre, Niegocin, Śniardwy and Tałty. In some of these lakes, we collected *G. lacustris* without invasive species which suggests also the possible presence of *P. quadrispinosa* in these lakes. *Pallasiola quadrispinosa* thrives in colder temperatures and typically resides in deeper waters during the summer months, which may explain why the species was not recorded during our summer samplings.

Our findings report the set of lake features promoting the distribution of native amphipod species in the studied lakes. As the study area is highly impacted by tourist activities, our results can be useful for better comprehension of the threats to native amphipods in other regions with similar levels of anthropogenic pressure and biological invasions. Our conclusions may highlight the need to protect isolated lakes from tourism and urban area development.

Distribution of invasive amphipods

Freshwater NIS can easily spread with tourist activities, including yachting and angling in particular. Our results show that the number of boats is one of the factors which best explains the distribution of *D. villosus* (Fig. 5). The main part of the Masurian Lakeland with a high abundance of this species covers the area of high tourist activities, i.e. lakes from group I in the northern part of the Lakeland (Figs 2, 4). Yachting is a very significant component of tourism in the Masurian Lakeland, reaching 37% of total tourist activities in the region (Kulczyk et al. 2016). Masurian tourist routes run through these lakes, thus, tourist boat activity supplements yachting. In 2016, we recorded *D. villosus* in all these lakes. A good example is Lake Niegocin, which has a high level of tourist pressure and a rapid invasion of *D. villosus* was observed in 2016. In 2014, the species was absent in this Lake, while in 2016, it constituted 81% of all sampled amphipods. Lake Niegocin is located between the other lakes with high tourist pressure and the Masurian tourist route runs through this lake. The evidence of high tourist activity in this lake can be the high number of car parks per km of shoreline and one of the highest, amongst the Masurian lakes, number of beds in accommodation establishments in 2014 (Kulczyk et al. 2016).

Similar findings were done in other tourist lakes. In Alpine lakes, with higher yachting activity than in the Masurian Lakeland, the expansion of *D. villosus* was caused by yachting and using diving equipment (Baćela-Spychalska et al. 2013; Rewicz et al. 2017). Many species using boat biofouling to invade new waterbodies have broad tolerance to desiccation (Baćela-Spychalska et al. 2013; Glisson et al. 2020). Likewise, *D. villosus* has a high tolerance to air exposure (Rewicz et al. 2014). Moreover, the species is usually associated with another invasive species – zebra mussel (*Dreissena polymorpha*) and can survive up to six days out of the water between mussels fouling the boats (Martens and Grabow 2008). Similarly, the species can be transported with algae and macrophytes (Minchin et al. 2019). It enables them to expand rapidly in new waterbodies, including those isolated from others, by overland transport of boats and yachting equipment.

Overland transport of boats may explain the invasion of *D. villosus* in our study area. Although *D. villosus* was found in most of the recently studied lakes, the species was not found in the River Pisa and the River Węgorapa. These rivers connect the Masurian Lakeland with large rivers, where *D. villosus* is present. It suggests the possible expansion of this species in the Masurian Lakeland by overland transport apart from these rivers. In certain lakes, we did not record *D. villosus*. These lakes have no direct contact with the invaded lakes and low tourist activity almost excludes the possibility of overland boat transport. In contrast to the Alpine lakes, we did not expect diving and angling (using waders) equipment to play a significant role in invading isolated waterbodies by *D. villosus* in the studied area.

Another strong factor explaining the distribution of *D. villosus* is the distance from town. Our results show that this species occurs mainly in the lakes with towns nearby. Proximity to the town and tourist activities are correlated with each other. Most of the marinas are located in towns with well-developed tourist facilities. Indeed, the

proximity to the ports and marinas is an important factor in promoting the expansion of *D. villosus* (Minchin et al. 2019). Higher tourist activities in proximity to urban areas may explain the distribution of *D. villosus* in the study area.

Distribution of other invasive species in the Masurian Lakeland, i.e. *P. robustoides*, *D. haemobaphes* and *C. ischnus*, concerns mainly the lakes with less developed shoreline and rather low water quality (higher class of water status). Predominantly, they are present in lakes with different conditions compared to those where *D. villosus* was found (Figs 4, 5). *Dikerogammarus haemobaphes* is the first Ponto-Caspian invasive amphipod recorded in the Masurian Lakeland (Jażdżewski 2003). This species was recorded in most of the studied lakes, as well as in the Rivers Węgorapa and Pisa. The presence of this species in the Rivers Bug and Narew suggests its invasion in the Masurian Lakeland from the east – from the River Dnieper. Despite the broad distribution of *D. haemobaphes* in the lakeland, this species was quickly over-dominated by *P. robustoides*. The latter species was first recorded in the study area in 2007 (Jażdżewska and Jażdżewski 2008). Three hypothetical routes of *P. robustoides* invasion to this region were proposed – from Kaliningrad (Russia) via the Pregel and the Węgorapa Rivers; from Lithuania via the Augustów Canal; from the Baltic Sea via the River Vistula and its tributaries (Jażdżewska and Jażdżewski 2008). However, since the first record of *D. villosus* in the region in 2014, the abundance of both species – *D. haemobaphes* and *P. robustoides* – drastically decreased until 2016. In 2016, *D. villosus* became the most abundant species in the lakes studied. These results are not surprising as the latter species is a strong competitor and successfully eliminates other invasive and native amphipods (Dick and Platvoet 2000; Platvoet et al. 2007; Bącela-Spychalska et al. 2012; Rewicz et al. 2014; Mathers et al. 2023). Especially, two of them – *D. haemobaphes* and *P. robustoides* – are weaker competitors than *D. villosus*, occurring in different habitats and occupying different niches (Bącela-Spychalska et al. 2012; Kobak et al. 2016; Poznańska-Kakareko et al. 2021; Copilaş-Ciocianu and Sidorov 2022). PCA and CAPSCALE results did not show a strong pattern in the distribution of these species, contrary to *D. villosus*, which suggests that *D. haemobaphes* and *P. robustoides* avoid niches occupied by *D. villosus* (Figs 4, 5). *Dikerogammarus haemobaphes* and *Pontogammarus robustoides* have high desiccation resistance, enabling their overland transport with vessels (Poznańska et al. 2013). Although sailing and angling activities may play an important role in their spreading (Bącela-Spychalska 2016; Csabai et al. 2020), tourist activities probably play a minor role in their distribution in the Masurian Lakeland (Fig. 5).

Another species rapidly spreading in the Masurian Lakeland is *Chaetogammarus ischnus*. In the study area, this species was recorded for the first time in 2014 (Fig. 2A; Suppl. material 1). In two years, its increasing abundance coincided with the decline of the abundance of *D. haemobaphes* and *P. robustoides* (Fig. 2B). In 2016, *C. ischnus* constituted more than half of the collected individuals in Lake Śniardwy. An especially high abundance of this species was observed in the southern group of lakes (group II), contrary to *D. villosus* occurring mainly in the northern group (group I). Moreover, we recorded *Chaetogammarus ischnus* in the River Pisa and did not record this species in the River Węgorapa. These results may suggest that *C. ischnus* invaded the Masurian

Lakeland from the southern direction, i.e. from the River Narew and then via the River Pisa. However, in the lakes where we recorded *D. villosus* and *C. ischnus* co-occurring, the abundance of both species increased. *Chaetogammarus ischnus* usually occupies similar habitats to *D. villosus*, i.e. sites with hard substrate, particularly covered by *D. polymorpha* (Żytkowicz and Kobak 2008; Copilaş-Ciocianu and Sidorov 2022). The co-existence of both species may be attributed to the small body size of *C. ischnus*, which enables this species to occupy microhabitats without interfering with *D. villosus* (Borza et al. 2018). This microhabitat-scale differentiation allows for both species to exist within the same habitat. Between *C. ischnus* and *P. robustoides*, the habitats also overlap, but usually *P. robustoides* limits the occurrence of *C. ischnus* because of its larger body size and more predatory diet (Żytkowicz and Kobak 2008). Therefore, we can hypothesise that *D. villosus* eliminates *P. robustoides* in the lakes studied and then *C. ischnus* refills the empty niche. In several lakes, for example, Dargin and Kisajno, we observed that *C. ischnus* reached a similar abundance in 2016 as *P. robustoides* had in 2014 (Fig. 2B; Suppl. material 1). Similar rapid invasion of *C. ischnus* and elimination of native species was observed in the Great Lakes in the USA (Dermott et al. 1998) and River Rhine in Europe (Van der Velde et al. 2000), where rapid range extension of *D. villosus* was observed as well (Bollache et al. 2004). *Chaetogammarus ischnus* can disperse over great distances (Witt et al. 1997). This species is capable of utilising natural water connections between different water-bodies, but it can also be transported through shipping (Nalepa et al. 2001). Witt et al. (1997) noted that the euryhaline nature of the species enables it to be transported even via ballast waters. However, the understanding of the invasion process of *C. ischnus* is limited and demands further studies.

Our results constitute an important contribution to the long-term observation of expansion dynamics of Ponto-Caspian amphipods and can be part of global databases monitoring invasive species. Rapid expansions underline the importance of regular, annual samplings in lakes and watersheds connecting them with invasion corridors. We show the very important role of tourist activities in lakes in the expansion of alien amphipods, in particular of *D. villosus*. These findings underline the important role of permanent monitoring of yachting and shipping vessels. Our predictions can be applicable in other tourist freshwater areas and help designate protection zones limiting boating. Our results can be also valuable to studies on other biofouling taxa. The significance of the town's proximity for the amphipod invasion is due to well-developed tourist facilities in urban areas and possibly water pollution; thus, lakes shorelines and marinas should be controlled as well. Water connections between lakes also should be regularly monitored. Our records of *Chelicorophium curvispinum* in the River Narew in 2014 and 2016 suggest that this species may be the next recorded invasive amphipod in the Masurian Lakeland (see Suppl. material 1). Some studies show a rapid expansion of *C. curvispinum* in freshwater ecosystems with the presence of *Dreissena polymorpha* and shipping (Van den Brink et al. 1993; Jażdżewski and Konopacka 2002). The current distribution of other invasive amphipods, for example, *Obesogammarus crassus* and *Gammarus tigrinus*, suggest no direct risk of their expansion in the Masurian Lakeland soon, but permanent monitoring of their expansion is necessary.

Limitations of our study

Although our data come from several years, it is important to indicate that the most recent data come from 2016; thus, the current invasion status in the study area can be worse than what we present here. The lack of lakes where native and invasive amphipods co-occur makes some of our findings difficult to interpret and partially speculative.

One of the crucial findings of our study is the impact of boating on the invasions. However, we need to remember that the methods we used have some limitations. We used the maximum capacity of marinas as the number of boats in use. Although on busy days the percentage of used boats in the total number of moored boats is high, as shown in Ros et al. (2013), these data are not precise and might be an under-representation of reality. We need to remember that, the association between boat density and the propagation of invasive amphipods has not been established through direct observation of vessel biofouling communities.

Lakes, especially those with high shoreline complexity, provide many habitats which various species can occupy. Therefore, analysing the data based on one sampling per lake may not depict the real diversity of the amphipod communities. Especially, the lack of records of amphipods in Lake Nidzkie, which has a connection with other lakes and Masurian tourist routes, suggests not enough efficient sampling. We need to be aware that the absence of a species in one sample does not exclude the possible occurrence of this species in other habitats of the same lake. Thus, our results, showing the replacement of native species by invasive species, present interesting trends, but are insufficient to conclude the extinction of certain species. Therefore, our findings should be perceived as predictions, not postulates.

Future directions

Future studies would benefit from utilising a more thorough and systematic sampling to provide a more accurate and reliable picture of the invasion process.

Our results show the importance of the proximity of sampling points to towns for invasions. Although we assume that this correlation is connected with tourist facilities and pollution, implementation of more data is needed in the future. Particularly, the distance between sampling points or lake centroid and marinas should be implemented in the analyses (Cole et al. 2019; Minchin et al. 2019). A significant effect of distance to marinas on invasions was noted by Minchin et al. (2019). Marinas are critical entry points for many invasive species and may play the role of reservoirs for newly-introduced invaders (Glasby et al. 2007; Ros et al. 2013; Fernández-Rodríguez et al. 2022).

Additionally, using the actual number of boats in use in the area would be advisable as was done in studies by Bącela-Spychalska et al. (2013) and Keramidis et al. (2018). Moreover, our knowledge about what part of the vessels are fouled by amphipods, which particular species can be transported and on what maximum distance is still scarce. Future studies would also benefit from including inspections of boats and ropes to identify potential vectors for amphipods, such as algae and mussels.

Dikerogammarus villosus, *Chaetogammarus ischnus* and *Chelicorophium curvispinum* can be transported with zebra mussels. Therefore, it is advisable to incorporate data on the occurrence of *D. polymorpha* in lakes and on vessels for future research. The type of vessel can also be an important factor. For instance, motorboats can be vectors of invasions, while canoeing does not play this role (Venohr et al. 2018). In this context, the presence/absence of silent zones, i.e. lakes or their parts where using boats with motors is forbidden, should complete the analyses. Knowledge about the success of the “check, clean and dry” strategy in the study area is missing. We expect that none of these methods is implemented as the local law does not demand their respecting. The method to prevent transporting invasive species on boats, as described in Mohit et al. (2021), should be tested in the Masurian Lakeland. Surveys amongst fishermen and tourists are worth collecting and analysing (Cole et al. 2019). To gain deeper insights into these dynamics, we recommend the establishment of an inter-lakes traffic registry. This registry would provide crucial data regarding boat traffic and potential pathways for the introduction of invasive species. Prevention measures and facilities for anglers should also be studied (Smith et al. 2023). To better understand which species can be transported by vessels, it is important to experimentally test the resistance of different invasive species like *C. ischnus* and *C. curvispinum* to desiccation.

As far as the financial and technical situation allows, samples should be collected from a large number of points on each lake. Additionally, studying a greater set-up of lakes would allow better tracking of invasions and more accurate detection of all amphipod species in the lakes. Finally, tracking of the invasion process can be supported by molecular studies (e.g. Mamos et al. (2021)).

Conclusions

The rapid expansion of the invasive Ponto-Caspian amphipods observed in this study aligns with a general trend along European freshwater basins. The contraction of the range and niche of native species when faced with more aggressive (e.g. *D. villosus*) and/or generalist (e.g. *C. ischnus*) species is something expected and confirmed by our findings. Even though many lakes seem to be still free from amphipod invaders, this may be for a short time considering the abrupt increase we have registered in just two years.

Our study emphasises the need for a comprehensive approach to understanding and addressing the dispersal of alien species through human activity. Our findings highlight the important role of boats in the spread of invasive amphipods within lake systems. The invasion process of *Dikerogammarus villosus* especially suggests the possible impact of overland boat transport in spreading this species in new lakes.

Furthermore, it is essential to raise awareness amongst lake users about the negative consequences of biological invasions and the necessity of implementing a “check, clean and dry” policy. By educating and engaging lake users, we can foster a sense of responsibility and cooperation in preventing the spread of invasive species. Implementing these measures collectively will contribute to better biosecurity practices and safeguard the ecological integrity of lakes against invasive species.

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Supplementary material I

Metadata for each sampling site and date

Authors: Krzysztof Podwysocki, Andrea Desiderato, Tomasz Mamos, Tomasz Rewicz, Michał Grabowski, Alicja Konopacka, Karolina Bącela-Spychalska

Data type: xlsx

Explanation note: Sampling sites between the years 2001–2016 with a number of individuals (or +/-) for the presence/absence) of each recorded Amphipoda species (names of invasive species have been underlined). Symbols for lakes provide two-letter acronyms used on the figures. Symbols for rivers and canals provide three-letter acronyms used on the figures. Water QS: Water quality status (Soszka et al. 2016); A/V ratio: Surface area to volume ratio (Soszka et al. 2016); Shoreline development: Shoreline length to surface area ratio (<https://mojemazury.pl>); Density of boats: number of boats per ha of lake surface (<https://mazury24.eu>; <https://skorupki.mazury.info.pl>).

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Supplementary material 2

Summary of the best-fitting Bernoulli GLMM for the presence of native gammarid – *Gammarus lacustris*

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Data type: docx

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Supplementary material 3

Results of PERMANCOVA test using 9999 permutations

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Data type: docx

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Supplementary material 4

Supplementary image

Authors: Krzysztof Podwysoki, Andrea Desiderato, Tomasz Mamos, Tomasz Rewicz, Michał Grabowski, Alicja Konopacka, Karolina Bącela-Spychalska

Data type: jpeg

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Competitive interaction in headwaters: slow upstream migration leads to trophic competition between native and non-native amphipods

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Abstract

The spread of non-native species is one of the outcomes of global change, threatening many native communities through predation and competition. Freshwater ecosystems are particularly affected by species turnover with non-native species. One species that has been established in Central Europe for many decades – or even a few centuries – is the amphipod crustacean *Gammarus roeselii*. Although *G. roeselii* is nowadays widespread in major river systems, there have been recent reports of its spread into smaller streams that are typically inhabited by the native species *Gammarus fossarum*. Due to their leaf shredding ability, *G. fossarum* takes up a key position in headwater streams. This raises the important question, to what extent *G. roeselii* can equivalently take over this function. To answer this question, we collected both species from nine different sites in a mid-mountain river system (Kinzig catchment, Hesse, Germany) and investigated their functional similarity using a combination of stable isotope analysis, gut content and functional morphology. The species hardly differed in morphological characteristics, only females showed differences in some traits. Gut content analysis indicated a broad dietary overlap, while stable isotopes showed a higher trophic position of *G. roeselii*. The observed functional overlap could intensify interspecific competition and allow the larger and more predaceous *G. roeselii* to replace *G. fossarum* in the future as a headwater keystone species. However, the differentiation in the stable isotopes also shows that co-existence can occur by occupying different trophic niches. Moreover, the wide range of inhabited sites and exploited resources demonstrate the omnivorous lifestyle of *G. roeselii*, which is likely to help the species succeed in rapidly changing environments.

Keywords

Freshwater ecosystems, functional morphology, *Gammarus*, gut content analysis, stable isotope analysis, trophic niche

Introduction

The introduction and spread of non-native species pose a threat to native communities globally (Gallardo et al. 2016). Due to their interconnection, streams are particularly affected by invasive species (Bij de Vaate et al. 2002; Leuven et al. 2009), which can cause a decrease of native species through predation and competition (Van der Velde et al. 2000). This change is not only expressed on a taxonomic level (Van der Velde et al. 2009), but probably also leads to changes in functional characteristics of ecosystems (Rosenfeld 2002). Non-native species might possess traits and behaviour that enable them to fulfil similar ecological functions as the native species they displace. However, the non-native species did not evolve within the recipient ecosystem and might lack natural predators, competitors or parasites that would otherwise regulate their populations in their native range (Torchin et al. 2003; Sih et al. 2010). Consequently, this unregulated population growth can lead to changes in resource availability and disrupting ecological interactions. However, post-invasion transformations of community structure and ecosystem function can differ immensely, are context-dependent and, often, poorly understood (Parker et al. 1999; Strayer et al. 2006; Kenis et al. 2009; Jeschke et al. 2014; Kumschick et al. 2015; Bellard et al. 2016).

A key group that is currently enormously affected by species-turnover is the taxonomic group of amphipods (Jażdżewski 1980; Leuven et al. 2009). Native *Gammarus* spp. are key species in freshwater ecosystems, due to their role in the decomposition of organic matter – an essential process in the headwaters of stream ecosystems (Cummins and Klug 1979; Vannote et al. 1980; Gessner et al. 1999; Graça 2001). However, the species turnover that is currently attracting attention is taking place in larger rivers (Leuven et al. 2009; Jourdan et al. 2016), while small headwaters – which are often not monitored within the Water Framework Directive – receive far less attention. While *Gammarus fossarum* Koch, 1836, is a typical headwater species, *Gammarus roeselii* Gervais, 1835, mainly inhabits the downstream parts of the rivers (Pöckl and Humpesch 1990; Pöckl et al. 2003). The exact origin and classification (native or non-native) of *G. roeselii* is still under debate, but the reduced genetic diversity (Csapó et al. 2020) and the main distribution on the Balkan Peninsula (Grabowski et al. 2017; Kabus et al. 2023) suggest that *G. roeselii* is probably an older invader in northern and western river systems outside the Danube system (Jażdżewski and Roux 1988). The species has been described near Paris in 1835 (Jażdżewski and Roux 1988), probably from a non-native population and has been spreading in Western Europe in the past centuries (Jażdżewski 1980; Jażdżewski and Roux 1988; Csapó et al. 2020). Nowadays, we observe an increasing spread of *G. roeselii* into smaller tributaries and upstream regions (Jourdan et al. 2019). In the Kinzig catchment (Hesse, Germany), some first order streams are

already colonised by *G. roeselii*, while others are still inhabited by *G. fossarum*. Interestingly, there are also streams where both occur syntopically. The similar phenotypic adaptation along environmental gradients in both species (Jourdan et al. 2019; Grethlein et al. 2022) now raises the question to which extent they share a similar trophic niche.

Investigating the trophic ecology of invasive species is necessary to gain a better understanding of the community-wide effects of invasions (Tillberg et al. 2007). Studies have shown that invaders across the animal kingdom often have a flexible and generalist diet (Garton et al. 2005; Tillberg et al. 2007; Caut et al. 2008; Zhang et al. 2010; Grey and Jackson 2012; Jackson et al. 2017; Mothapo and Wossler 2017). Especially in changing environments, omnivorous species or species with a broad trophic niche are more successful than those with a more specialised diet, so that omnivorous invasive species may prey on or compete with native species (Barbosa and Castellanos 2005; Simberloff 2010; Jackson et al. 2017).

The ecological niche of an organism is connected with its functional morphology (Bock and von Wahlert 1965). Therefore, morphological traits can provide additional important ecological insights (Premate et al. 2021). Characterising such traits – like body size and mouthparts – is suitable to evaluate the relationship between morphology and trophic ecology, which, in turn, can be tested against further results of stable isotope and gut content analyses (Premate et al. 2021). Morphological analysis combined with stable isotope analysis can be used to elucidate potential relationships between morphology and function (Hutchins et al. 2014), since trophic levels characterise the functional role of organisms (Hairston and Hairston 1993). So far, the relationship between morphology and ecology is not yet fully understood in many invertebrate groups and only now starting to become clearer in amphipods (Copilaş-Ciocianu et al. 2021).

Stable isotope analyses are useful for answering general questions about trophic structure (Vander Zanden et al. 1999; Post et al. 2000). However, this analysis is not able to distinguish very well between closely-related food sources with a similar isotopic value (McCutchan et al. 2003; Hood-Nowotny and Knols 2007; Bowes and Thorp 2015). Therefore, to gain qualitative information necessary to interpret the isotopic results, stable isotopes should be used together with other information, such as the analysis of the gut content (Inger and Bearhop 2008). This analysis has, in previous studies, been demonstrated to have a strong correlation with stable isotope data and, thus, can be considered a reliable reflection of diet in amphipods (Bacela-Spychalska and Van Der Velde 2013; Aumack et al. 2017). Copilaş-Ciocianu et al. (2021) identified a strong relationship between morphological traits and the proportion of food items found in the gut. These correlations can provide an important understanding of the functional morphology of freshwater amphipods (Copilaş-Ciocianu et al. 2021).

Stable isotope analysis is a common method used to reveal the trophic position of organisms in the field (Peterson et al. 1986; Cabana and Rasmussen 1996; Vander Zanden and Rasmussen 1999; Vander Zanden et al. 1999; Post et al. 2000; Post 2002; Layman et al. 2007). This analysis is an important tool to study food webs and has already been successfully used to detect the impact of biological invasions on trophic

structures (Vander Zanden et al. 1999; Van Riel et al. 2006; Mancinelli and Vizzini 2015; McCue et al. 2020). For nitrogen, isotope ratios ($^{15}\text{N}/^{14}\text{N}$; $\delta^{15}\text{N}$) of a consumer are on average 3 to 5‰ higher than of dietary items and increase with successive trophic levels (DeNiro and Epstein 1981; Minagawa and Wada 1984; Peterson and Fry 1987; Vander Zanden and Rasmussen 1999; Layman et al. 2007). On the contrary, the ratios of carbon isotopes ($^{13}\text{C}/^{12}\text{C}$; $\delta^{13}\text{C}$) show only minor changes since carbon moves through the food web with little alteration (DeNiro and Epstein 1978; Rounick and Winterbourn 1986; Peterson and Fry 1987; Layman et al. 2007).

We hypothesise that, in the focal study area, *G. fossarum* and *G. roeselii* share a similar trophic niche. This equivalence is suggested by a laboratory experiment where *G. roeselii* showed the same leaf consumption rate as *G. fossarum* (Jourdan et al. 2016). Moreover, we expect a high level of competition amongst ecologically similar species, which should lead to a niche shift in co-occurrence scenarios. Finally, *G. roeselii* is found both in large lowland rivers, but also occasionally in upper stretches, while *G. fossarum* is restricted to upper stretches (Pöckl and Humpesch 1990; Pöckl et al. 2003). Given that headwaters mainly receive energy input through terrestrial organic material, resulting in simpler food webs, while lower stretches exhibit more complexity due to increased nutrient availability and primary production (Vannote et al. 1980), we hypothesise that *G. roeselii* has a broader trophic niche compared to *G. fossarum*.

Materials and methods

Fieldwork

Gammarus fossarum and *G. roeselii* were collected with the kick-sampling method on 10 and 11 August 2021 at nine sampling sites in the Kinzig catchment in Hesse, Germany (Table 1; Fig. 1). The sampling sites and their site IDs correspond with the sites from Weigand et al. (2020). Adult and juvenile individuals, as well as submerged leaves of *Alnus* sp. or *Corylus* sp. (depending on availability), were collected at each sampling site. Gammarids displaying visible parasitism, such as acanthocephalans (Médoc et al. 2011;

Table 1. The nine sampling sites within the Kinzig catchment. The site ID (according to Weigand et al. (2020), name of the stream, the collected species and the GPS coordinates are given for each sampling site.

Site ID	Stream	Species	GPS
1	Gründau	<i>G. fossarum</i> + <i>G. roeselii</i>	50°14.93'N, 9°9.33'E
5	Bracht	<i>G. fossarum</i> + <i>G. roeselii</i>	50°22.62'N, 9°16.22'E
6	Bracht	<i>G. fossarum</i> + <i>G. roeselii</i>	50°26.22'N, 9°16.43'E
7	Salz	<i>G. roeselii</i>	50°25.00'N, 9°21.80'E
10	Ulm bach	<i>G. fossarum</i> + <i>G. roeselii</i>	50°20.18'N, 9°25.70'E
20	Schwarzbach	<i>G. fossarum</i> + <i>G. roeselii</i>	50°21.53'N, 9°33.11'E
33	Kinzig headwater	<i>G. fossarum</i> + <i>G. roeselii</i>	50°18.87'N, 9°36.98'E
37	Haselsbach	<i>G. fossarum</i>	50°13.87'N, 9°22.21'E
105	Riedbach, Kinzig	<i>G. fossarum</i>	50°22.62'N, 9°31.58'E

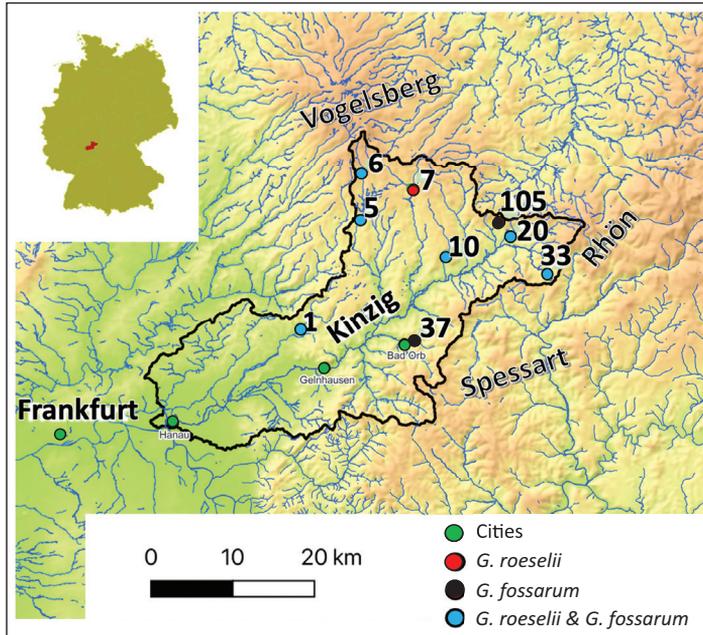


Figure 1. The nine sampling sites within the Kinzig catchment.

Kochmann et al. 2023), were omitted from the analysis due to their potential to impact the feeding behaviour and metabolic processes of the organisms.

Morphological and gut content analysis

The methodology largely followed Copilaş-Ciocianu et al. (2021). The animals were first soaked overnight in a 2% lactic acid solution and transferred to a 1:1 solution of 70% ethanol and glycerine. Dissections were performed in glycerine with the help of fine needles and microsurgical scissors. Appendages were glycerine-mounted on microscope slides. Photographs were taken using a Nikon DS-Fi2 camera attached to a Nikon Eclipse Ci-L microscope or a Nikon SMZ1000 stereomicroscope. Measurements were taken with Digimizer software (<https://www.digimizer.com/>), based on the photographs. A total of 15 female *G. fossarum*, 12 male *G. fossarum*, 14 female *G. roeselii* and 12 male *G. roeselii* were used for this analysis. We measured 35 functional morphological traits that mainly reflect the diet (body length, mouthparts, stomach and shape of gnathopods). Additionally, traits, indirectly related to diet that reflect sensory function (antennae) and locomotion (pereopods), were measured as well. For an overview of landmarks and function of these traits, see Copilaş-Ciocianu et al. (2021). The same individuals used for the morphological analysis were used for the gut content analysis. The gut content analysis followed the protocol of Copilaş-Ciocianu et al. (2021), using a Nikon Eclipse Ci-L microscope. In short, the gut was emptied out and evenly spread on a glass slide with a square grid (24 × 24 mm) containing 10 × 10

smaller squares. The gut content was classified into six categories (alga, arthropod, detritus, fungus, plant, sand) and the number of squares on which a particular food item occurred were counted to calculate proportions.

Stable isotope analysis

Two gammarids within each 2 mm size class were used for the analysis. Amphipods with a body size lower than 7 mm were considered juvenile. In total, 26 juveniles, 18 females and 24 males of *G. fossarum* and 19 juveniles, 36 females and 28 males of *G. roeselii* were used. Three leaf replicates per site were used as baseline for trophic position estimates. All samples were sorted, washed with distilled water and dried for 48 h at 60 °C. Afterwards, they were ground to a fine powder with a pestle and mortar. The powder for each amphipod sample was aimed to be 1 mg. In case juveniles did not have a high enough body mass, a composite sample of more individuals of the same species, sex and size class was used. The aimed weight for the leaves was between 3 mg and 4 mg powder for each sample. Carbon and nitrogen stable isotope ratios were measured at the Isotopic Research Laboratory of the Centre for Physical Sciences and Technology in Vilnius, Lithuania. Here, an elemental analyser coupled to the isotope ratio mass spectrometer (EA-IRMS, Flash EA1112–Thermo V Advantage) via the ConFlo III interface was used for the measurement.

In our amphipod samples, the C:N mass ratio varied over 3.5 and in our leaf samples the C exceeded 40%; thus, we corrected the $\delta^{13}\text{C}$ values for lipid content using the relevant formulae for aquatic animals and plants from Post et al. (2007). We referenced the trophic position of amphipods to ΔC and ΔN in each site by subtracting the corresponding mean values of the leaves. We further divided the ΔN by the conventional trophic fractionation factor value of 3.4‰ (Post 2002) and added one (baseline trophic level) to obtain the amphipod trophic level (TL) estimates.

Statistical analysis

To correct for body size, the morphological measurements were first regressed against the body length and the residuals across all sampling sites were pooled into four species by sex groups (female *G. roeselii*, male *G. roeselii*, female *G. fossarum*, and male *G. fossarum*). The mean value of residuals was used in case of missing values. The gut content data of all sampling sites were grouped in the same manner. Subsequently, principal component analyses (PCA) were conducted in PAST 4 (version 4.08; Hammer et al. 2001). They were computed with a correlation matrix. To test for dietary or morphological differences amongst groups, a one-way permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations and Euclidean similarity index was conducted in PAST. The PERMANOVA was performed either on the gut content data or all morphological traits combined or separately on four trait-complexes reflecting sensorial function (antennae, six measurements), food grasping and manipulation (gnathopods, eight measurements), food processing and digestion (mouthparts and

stomach, four measurements) and locomotion (pereopods along with their accompanying bases and coxae, 15 measurements). To test for differences between gammarids occurring alone compared to co-occurrence, PERMANOVA was performed on gut content and all morphological traits between those sites. Bonferroni correction was applied for further multiple comparisons between group pairs.

We analysed the difference in trophic position between species by building linear mixed-effects models (LMEMs) for each of the referenced metrics (ΔC and TL). In these models, we considered the interacting fixed effects of size, species and the syntopy (or co-occurrence) factor, while site was included as a random factor. The continuous size variable was centred around the global mean of 8.25 mm for more proper effect testing (but back-transformations were applied for the provided visuals). The effects were tested using type III analysis of variance with Satterthwaite's approximation for denominator degrees of freedom. These analyses were conducted by employing the R packages *lme4* v. 1.1-32 and *lmerTest* v. 3.1-3 (Kuznetsova et al. 2017) and visualised using the package *visreg* v. 2.7.0 (Breheny and Burchett 2017). We further conducted the *post hoc* group comparisons at minimum and maximum amphipod sizes with the Šidak *p*-value adjustment for eight tests using the package *emmeans* v. 1.8.8 (Lenth 2023). To test for the differences in ΔC and TL between sexes, we extracted the residuals from the LMEMs for the adult observations only and applied simple linear models with backward stepwise removal of terms (based on Akaike Information Criterion), starting with fully interacting effects of species, sex and syntopy.

In the bivariate stable isotope space, we approximated the population isotopic niches as ellipses containing 95% of the data with their area estimates ($BEA_{95\%}$). We also estimated the overlaps between the species in the six syntopic sites and standardised them as proportions of the sum of the non-overlapping ellipse areas (0 – no overlap, 1 – complete overlap). For this, we used the Bayesian estimation available in R package *SIBER* v. 1.2.7 (Jackson et al. 2011) and provided the estimates as modes with 95% credible intervals of the posterior distributions (400 draws). Using these draws, within each of the six syntopic sites, we tested if: (1) the $BEA_{95\%}$ of *G. roeselii* is wider than the ellipse of *G. fossarum*, (2) if the overlap is larger than 0 and (3) if the overlap is larger than 60% which is the commonly considered threshold for an ecologically significant overlap (Jackson et al. 2011). Provided probabilities were translated to be treated conventionally – significant effects when $p < 0.05$.

Results

Morphological analysis

Results from omnibus PERMANOVA testing for all traits ($F = 12.7$, $p = 0.001$) revealed a significant morphological differentiation between sexes within *G. roeselii* and within *G. fossarum* and between females of both species, but not males (see Suppl. material

Gut content analysis

The PCA of the gut content indicated that the most important differentiation between specimens was amongst the detritus, sand vs. plant axis, explaining 29.9% of variation (Fig. 3). The second axis of differentiation was between detritus and sand and it explained 21.4% of the variance (Fig. 3). The PERMANOVA revealed no significant differentiation between species or sexes when only considering co-occurrences ($F = 1.3$, $p = 0.24$). All groups overlapped to a great extent (Fig. 3). Results from PERMANOVA testing for gut content between syntopic and non-syntopic populations did reveal a significant differentiation between *G. fossarum* alone/co-occurring with *G. roeselii* ($F = 3.6$, $p = 0.032$), but not between *G. roeselii* alone/co-occurring with *G. fossarum* ($F = 2.6$, $p = 0.065$, Fig. 3b).

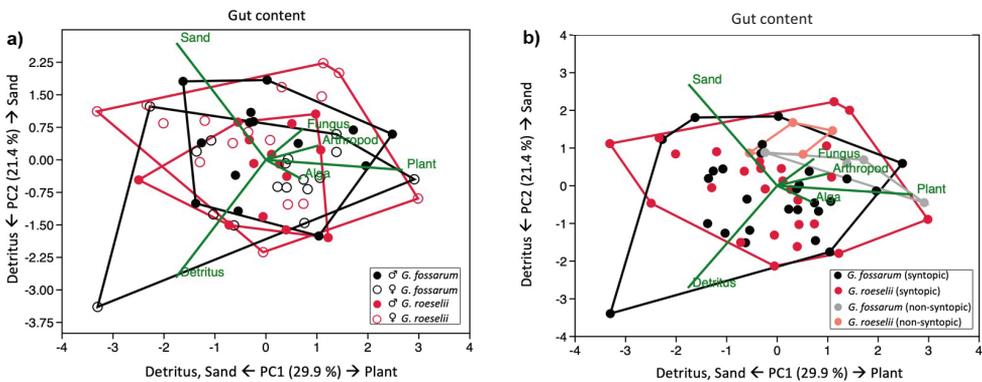


Figure 3. A principal component analysis (PCA) scatterplot depicting dietary differentiation with respect to the proportion of various food items of species by **a** sex and **b** by non-syntopic and syntopic occurrence (sexes combined).

Stable isotope analysis

Isotopic position

The results of stable isotope analysis revealed pronounced niche differentiation between *G. fossarum* and *G. roeselii*, with *G. roeselii* generally occupying a higher trophic level (Fig. 4). At most sites, ΔC values varied indicating ^{13}C -enrichment of amphipods relative to the tree-leaf detritus. However, we observed a notable separation of site 7, where *G. roeselii* was exceptionally ^{13}C -depleted relative to the leaves (coincidentally, *Alnus* sp. leaves were missing at the site, thus *Corylus* sp. were used). Trophic level of amphipods mostly fell within the normal range between herbivory (TL = 2) and complete carnivory (TL = 3), with the extremes of 1.8 in *G. fossarum* juveniles from site 37 and slightly above 3.0 in the largest adults of *G. roeselii* from site 6 and site 10. The trend of increasing TL with size was also reflected in the data. Estimates of trophic

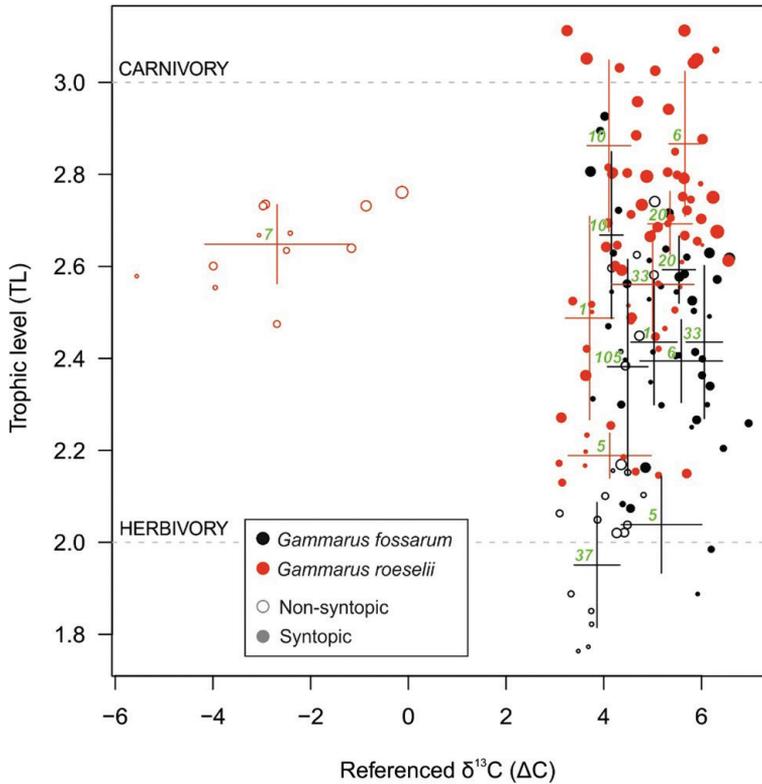


Figure 4. Referenced stable isotope biplot of studied amphipods showing trophic niches by site (means \pm SD). Point size reflects animal body size. Green labels at the means correspond to site IDs from Table 1.

position for different amphipod size groups derived from stable isotope analysis are provided in Suppl. material 1: table S2.

Both LMEMs of ΔC and TL (Table 2) returned a significant second order interaction of amphipod size, species and the syntopy factor, indicating variable steepness of the ontogenetic slopes of gammarids. Judging by the various modelled cases (Fig. 5), there was always an increasing ΔC and TL trend with amphipod size and *G. roeselii* generally tended to exhibit lower ΔC and higher TL values than *G. fossarum*. Regarding both metrics, the interspecific differences in the overall position, as well as the slopes, were more evident across the non-syntopic sites, while, when co-occurring, the two species tended to overlap more and to exhibit very similar ontogenetic slopes. Across the syntopic sites, both amphipods appeared to have high ΔC values, similar as in *G. fossarum*-only sites and their juveniles converged more to intermediate TL values. The ΔC slope of *G. roeselii* and TL slope of *G. fossarum* were visibly steeper across the non-syntopic sites in comparison to the syntopic ones, suggesting wider ontogenetic niches in respective metrics when a single species is found. (It is important to note that, to some extent, the observed patterns may have been biased by the single *G. roeselii*-only site coinciding with a more degraded habitat.)

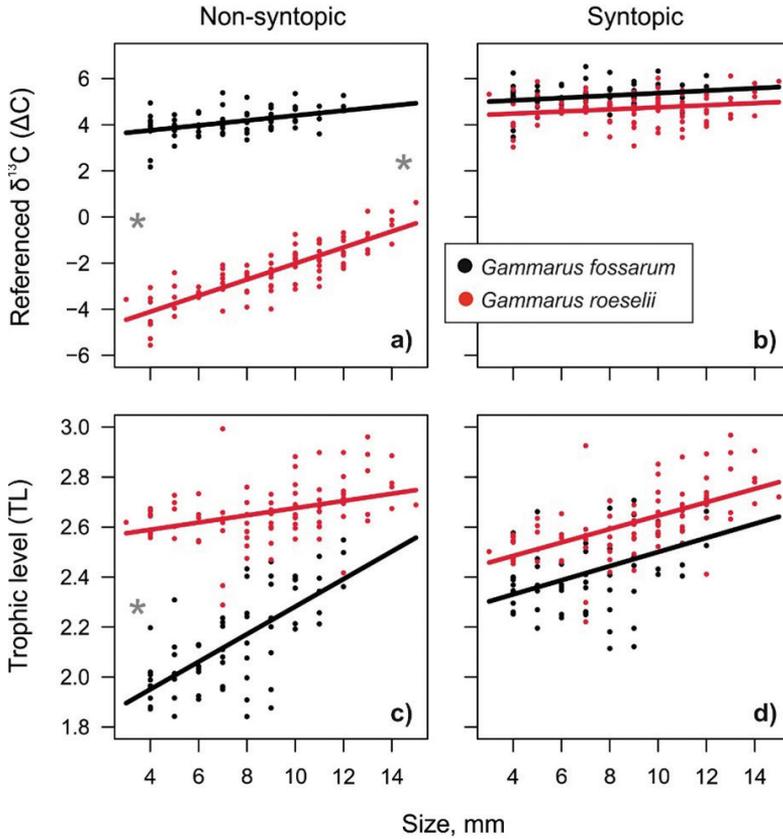


Figure 5. Effects of amphipod size, species and their syntopic occurrence on isotopic metrics of trophic position within the linear mixed-effects models of **a, b** referenced $\delta^{13}\text{C}$ (ΔC) and **c, d** of trophic level (TL) by **a, c** non-syntopic vs. **b, d** syntopic sites. See Table 2 for effect tests. Grey asterisks between species' lines indicate significant ($p < 0.05$) interspecific differences at corresponding size extremes according to *post hoc* analysis. Not shown here, but this analysis also indicated significant ΔC differences within the smallest and largest specimens of *G. roeselii* occurring in non-syntopic vs. syntopic sites.

Table 2. Results of analysis of variance (type III decomposition) from the linear mixed-effects models of isotopic metrics of trophic position – referenced $\delta^{13}\text{C}$ (ΔC) and trophic level (TL) – testing for the interacting effects of amphipod size, species (*Gammarus fossarum* vs. *G. roeselii*) and their syntopic occurrence. See Fig. 5 for effect plots.

Tested term	df	ΔC model			TL model		
		df _{denominator}	F	p	df _{denominator}	F	p
Size	1	142.6	35.2	< 0.001	142.1	43.4	< 0.001
Species	1	10.6	125.1	< 0.001	9.4	7.0	0.026
Syntopy	1	9.7	114.8	< 0.001	9.1	0.6	0.459
Size : Species	1	142.7	6.3	0.013	142.1	5.0	0.027
Size : Syntopy	1	142.6	14.7	< 0.001	142.1	0.6	0.444
Species : Syntopy	1	10.6	87.8	< 0.001	9.4	1.9	0.202
Size : Species : Syntopy	1	142.7	7.1	0.008	142.1	4.3	0.039

Regarding the effect of sex across the adult dataset, the stepwise procedure removed all the effects apart from sex from the linear model of ΔC and indicated a model without predictors for TL (although sex was removed last). Thus, we ended up applying simple t -tests using only the sex factor. These indicated a marginally higher female ΔC ($t_{104} = 1.9$, $p = 0.061$), but no effect of sex on TL ($t_{104} = 0.4$, $p = 0.69$).

Isotopic niche width and overlaps

Isotopic niche widths of the populations and the overlaps between species are provided in Table 3. Although the mode estimates of $BEA_{95\%}$ were often larger in *G. roeselii* than in *G. fossarum*, the Bayesian testing indicated such a pattern significantly only in site 10 ($p = 0.030$ as opposed to $p \geq 0.84$ in other cases). All the six overlaps were larger than 0 ($p \leq 0.037$), but none of them exceeded 60% ($p \geq 0.96$), indicating a relatively low trophic niche overlap.

Table 3. Population isotopic niche widths as ellipse areas ($BEA_{95\%}$) and their absolute and relative overlaps by study site. The Bayesian estimates are provided as modes and 95% credible intervals.

Site ID	<i>G. fossarum</i> $BEA_{95\%}$	<i>G. roeselii</i> $BEA_{95\%}$	Overlap	%Overlap
37	3.47 (1.56–6.59)	-	-	-
105	5.20 (3.01–9.21)	-	-	-
6	3.51 (2.10–6.79)	3.05 (1.96–5.37)	0.02 (0.00–1.44)	0.00 (0.00–0.20)
10	2.10 (0.99–4.07)	4.76 (3.09–9.63)	1.61 (0.46–2.88)	0.26 (0.08–0.49)
20	1.31 (0.66–2.94)	1.93 (1.34–3.67)	0.70 (0.00–1.47)	0.27 (0.00–0.55)
33	3.53 (2.09–8.38)	4.85 (3.10–8.40)	1.73 (0.15–3.17)	0.24 (0.05–0.39)
1	3.63 (1.56–9.25)	4.78 (1.79–11.19)	0.04 (0.00–3.30)	0.00 (0.00–0.32)
5	3.84 (1.94–10.98)	2.39 (1.42–4.85)	1.19 (0.00–2.50)	0.02 (0.00–0.40)
7	-	6.75 (3.66–12.41)	-	-

Discussion

Our study revealed a strong overlap in morphology and gut content between the native *G. fossarum* and non-native *G. roeselii* in headwater streams. However, stable isotopes indicated a stronger dietary differentiation between the two species when occurring alone and a more similar trophic niche when occurring together, with *G. roeselii*, however, generally occupying a higher trophic level. In addition, the gut content analysis confirmed that *G. fossarum* appears to have a different diet when occurring alone. This indicates that, despite their apparent functional morphological equivalence, the two species exploit different food resources. Below, we expand on the significance of these findings.

Overall, morphological differentiation occurs between the sexes rather than between species when looking at the combined data, pooled over all sampling sites. Only in some traits of the females (gnathopods, pereopods and coxae) could we see differences between the species. This differentiation between females of both species could be explained by different reproduction characteristics of the species, such as different thermal optima for maximum fecundity (Pöckl 1993). As reproduction involves a met-

abolic cost (Sutcliffe 1992), a different reproduction effort could mean that different energy levels are available for growth and, thus, translating into different sizes of morphological features. Besides, the size of males is significantly affected by sexual selection, while females are significantly affected by natural selection (Ward 1988). Sexual selection of a trait is induced by competition over mates (Andersson 1994), while natural selection is induced by trait variation amongst individuals (Endler 1986). The competition between males of both species might be similar throughout the sampling sites and, therefore, the males show similar morphological traits. On the other hand, females of both species experiencing natural selection might lead to morphological trait differentiation. Similar morphological features of males of both species point to an absence of morpho-functional differentiation, the occupation of a similar trophic niche and ecological similarity (Cothran et al. 2013; Fišer et al. 2015; Copilaş-Ciocianu et al. 2021). However, morphological similarity does not always mean ecological equivalence. Fišer et al. (2015) found ecological differentiation between four morphologically similar *Niphargus* species, which could potentially lead to different ecological roles in the ecosystem. Moreover, Premate et al. (2023) discovered that morphological traits are influenced not only by trophic position, but also by the specific habitat type. This observation could also explain the similar morphology of our study species occurring in the same headwater habitat.

The gut content analysis showed a strong dietary overlap between the species when pooled over all sampling sites. Our results thus indicate that the foraging on the same food sources might lead to competition between the two species. In principle, a strategy adopted by different amphipods to reduce competition for limited resources could be to utilise resources in different ways, in different microhabitats or at different times (Piscart et al. 2010, 2011; Mauchart et al. 2017; Premate et al. 2021). Apart from that, our species differed significantly regarding the $\delta^{13}\text{C}$ level, which has also been observed in other co-occurring freshwater amphipods (Premate et al. 2021). Such partitioning of food resources is expected amongst co-occurring species (Schoener 1974; Chesson 2000), facilitating a stable co-existence (Chesson 2000). Our analysis showed a significant differentiation in gut content for *G. fossarum* when occurring alone compared to co-occurring with *G. roeselii* and close to significance for *G. roeselii* when occurring alone compared to co-occurring with *G. fossarum*. This could be explained by their, in general, separated occurrence in different river sections (Pöckl and Humpesch 1990; Pöckl et al. 2003) and the different availability of resources in these sections (Vannote et al. 1980). On the other hand, the absence of dietary separation for co-occurring populations might be a consequence of high abundance of food during the study period and could indicate potential for interspecific competition (Piscart et al. 2011; Rothhaupt et al. 2014). Interspecific competition, in turn, can reduce intraspecific specialisation, therefore, reducing the niche breadth of individuals (Araújo et al. 2011), leading to a dietary overlap. In addition, dietary separations or overlaps can change frequently in populations depending on spatio-temporal availability of food items and on the abundance of competing species (Rothhaupt et al. 2014). Thus, stable isotope analysis is useful for studying the diet on a longer time-scale.

The stable isotope analysis revealed that both species had more similar trophic niches when they co-occurred. Specifically, *G. fossarum* underwent a trophic level increase while *G. roeselii* a decrease which was also accompanied by a shift in the carbon source. Although this result was unexpected and non-intuitive at a first glance, it could be explained by reciprocal predation on juveniles or recently moulted individuals of the other species, as observed in another native and non-native amphipod species pair (Dick et al. 1993; Dick 1996; Dick and Platvoet 1996). Moreover, other researchers have discovered niche segregation in co-occurring amphipods in order to facilitate co-existence and avoid competition (Piscart et al. 2011; Premate et al. 2021).

Nevertheless, our stable isotope analysis revealed that, even though both species experience a niche shift, *G. roeselii* still has generally a higher trophic position compared to *G. fossarum* when both species occur together, contradicting our first hypothesis of a shared trophic niche. One reason why we have not found this more carnivorous lifestyle in the gut content could be that animal material can be digested more quickly (Guerra-García et al. 2014), but the stable isotopes of animal prey are incorporated in the tissues of the amphipod and, thus, influence the stable isotope analysis. The higher trophic position of the non-native *G. roeselii* observed in our stable isotope analysis could be an advantage compared to the native *G. fossarum*. Animal material, a high quality and easily-assimilated food item, can improve the growth of *Gammarus* sp. (Anderson and Cummins 1979; Crenier et al. 2017). The acceleration of growth, maturation and reproduction progress when being fed animal matter has been observed for several *Gammarus* species (Vassallo and Steele 1980; Smith 1988; Delong et al. 1993). In addition, it has been observed for *G. fasciatus* that, with increasing size, the amount of animal matter found in the gut increased proportionally (Summers et al. 1997). The present study showed that larger individuals occupy a higher trophic niche, confirming an ontogenetic niche shift. Thus, since *G. roeselii* usually has a larger body length, it will presumably consume more animal material than the smaller *G. fossarum* (DeLong et al. 1993). In return, individuals of *G. fossarum* have a lower trophic position than *G. roeselii*. Since *G. fossarum* also has a smaller body size and smaller gnathopods than *G. roeselii*, it may be mainly limited to detritus because their smaller gnathopods may not be large enough to handle larger food items, such as animal matter (Summers et al. 1997). This is supported by a laboratory experiment of Delong et al. (1993), which showed that small individuals of *G. fasciatus* showed a delayed growth response when fed animal matter, but a normal growth when fed only leaf litter. However, *G. fossarum* is generally also known to be predatory and able to exploit animal food resources (Stoffels et al. 2011; Georgievová et al. 2020; Syrovátka et al. 2020) resulting in gammarids generally being classified as rather omnivorous (MacNeil et al. 1997).

Overall, our results indicate that *G. roeselii* exhibits a broader trophic niche than *G. fossarum* confirming our second hypothesis. It has been shown that, when *G. roeselii* co-occurs with *G. fossarum*, it significantly affects their micro-distribution (Mauchart et al. 2017). *G. roeselii* chooses habitats with macrophytes, (dead) wood or deposits of CPOM (coarse particulate organic matter), whereas *G. fossarum* prefers coarse gravel and cobbles (Mauchart et al. 2017). This suggest different sources of algal or terrestrial

$\delta^{13}\text{C}$ (Finlay 2001, 2004) for the diet of *G. roeselii* and could be an explanation for its broader trophic niche. Moreover, in our stable isotope analysis, population from site 7 appears to be an outlier with lower $\delta^{13}\text{C}$ values. Site 7, unlike the other sites, was highly morphologically degraded, canalised and without significant riparian vegetation. Unlike *G. fossarum*, *G. roeselii* is able to occur in many anthropogenically shaped habitats (Mauchart et al. 2014; Enns et al. 2023; Kochmann et al. 2023). Apparently, in such habitats, *G. roeselii* is able to exploit resources from different food chains, such as algae. On the other hand, the larger size of *G. roeselii* could be an explanation for its broader trophic niche, because the larger size can offer competitive advantage (Young 2004). This advantage arises from the potential for a wider dietary range as size increases, potentially resulting in accelerated growth rates and shorter reproduction times (Summers et al. 1997). Moreover, it enables the exploitation of seasonal changes of food items (DeLong et al. 1993). High trophic diversity is one of the most important factors responsible for the dispersal success of amphipods (Legeżyńska et al. 2012), because invasive species must be able to gain food material in heterogeneous habitats (Rothhaupt et al. 2014). Nevertheless, in a future study, it needs to be elucidated whether *G. roeselii* has a similar or different niche in their native range on the Balkan Peninsula. Presumably, *G. roeselii* could become even more competitive through depending on leaf litter as constant adaptation, effectively exploiting leaf litter, and also higher quality food sources and continuing to colonise tributaries and headwaters (Jourdan et al. 2016).

The omnivorous diet of both amphipods, the higher trophic niche of *G. roeselii* seen in our stable isotope analysis and the observed microhabitat partitioning in the field (Mauchart et al. 2017) can facilitate and explain the co-existence of both species. However, unlike *G. fossarum*, *G. roeselii* possesses dorsal pleosomal spines. An experimental study identified these as an effective defence mechanism against predatory fish (Bollache et al. 2006). The protection through spines can probably not only facilitate its spatial distribution, but may also favour the colonisation of new niches, hence increasing its invasion potential (Copilaş-Ciocianu et al. 2020). The low predation of *G. roeselii* may promote its establishment in communities with native species, such as *G. fossarum* (Bollache et al. 2006). Moreover, headwater sections of streams are particularly impacted by anthropogenic pollution and a subsequent species turnover (Betz-Koch et al. 2023; Enns et al. 2023). This ongoing pollution can facilitate the spread and establishment of *G. roeselii* into headwaters (Mauchart et al. 2014; Jourdan et al. 2024) and can lead to the decline of the more sensitive *G. fossarum* (Enns et al. 2023). In addition, temperature records for the herein studied sites show water temperatures between 14.4 °C and 21.5 °C, which are congruent with an earlier study of some of the sites (Grethlein et al. 2022). *G. roeselii* reaches sexual maturity faster, has a shorter brood development and a higher reproductive success than *G. fossarum* at temperatures above 12 °C (Pöckl et al. 2003). Furthermore, these characteristics of *G. roeselii* are enhancing with increasing temperature (Pöckl 1992). These life-history traits could facilitate invasion success and alter the community structure of invaded habitats (Grabowski et al. 2007; Rothhaupt et al. 2014). Thus, due to increasing water

temperatures associated with climate change and ongoing pollution through anthropogenic impact, *G. roeselii* might outcompete *G. fossarum* in headwaters in the future (Pöckl et al. 2003; Enns et al. 2023; Jourdan et al. 2024).

Conclusions

Our study revealed that the non-native *G. roeselii* is morphologically similar to the native *G. fossarum* in headwater streams. We also found similar food items in the gut content, which showed the generally omnivorous lifestyle of both species. However, stable isotopes indicated that the trophic niches of both species differ substantially, with *G. roeselii* being more predaceous and generally having a broader niche. This indicates that, despite their shared morphological characteristics and omnivorous tendencies, there is a noticeable niche differentiation in *G. roeselii*, consequently influencing the headwater food web. In situations of ample resource availability, co-existence between both species may be possible. However, in cases of resource scarcity, we expect *G. roeselii* to be competitively superior, particularly given its ability to exploit a broader range of food resources, regardless of their quality.

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Supplementary material I

Supplementary information

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Data type: docx

Explanation note: **table S1**. Results (p -values) of the PERMANOVA for the morphological traits of GR-F (female *G. roeselii*), GR-M (male *G. roeselii*), GF-F (female *G. fossarum*) and GF-M (male *G. fossarum*). PERMANOVA was performed with 9999 permutations and Euclidean similarity index. Bonferroni correction was applied for multiple comparisons between group pairs. Significant p -values (< 0.05) are marked in bold. **table S2**. Trophic position metrics of different amphipod size groups by study site (derived from stable isotope analysis relative to tree-leaf detritus). Provided values are means with standard deviations.

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